Attack and defence in cassava bacterial blight

Kemp, Benjamin Peter

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Attack and Defence in Cassava Bacterial Blight

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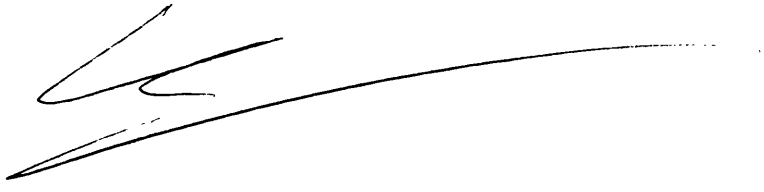

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Abstract

Cassava bacterial blight (CBB) is a devastating disease caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). CBB is a poorly understood disease and cassava resistance to CBB has yet to be characterised at any level. This study investigates three areas of the disease; pathogenicity factors of *Xam*, preformed defences of cassava and induced defences of cassava.

Extracellular polysaccharide (EPS) is a well-known pathogenicity or virulence determinant for a number of plant and animal pathogens. An EPS minus (EPS^-) strain of *Xam* was created by targeted gene disruption of *gumD*, a gene essential for EPS production in *Xanthomonas campestris* pv. *campestris*. When introduced into the apoplast *via* leaf infiltration EPS^- strains failed to spread from the point of initial infection and did not cause many of the symptoms associated with CBB. EPS^- *Xam* injected into the petiole of susceptible cassava plants failed to cause any symptoms, whereas wild type *Xam* caused severe wilting and eventual leaf death. Therefore, EPS appears to be a pathogenicity or virulence determinant of *Xam*. Pectate lyase (Pel) is reported to be involved in the pathogenicity of a number of fungal and bacterial plant pathogens. The production by *Xam* of Pel *in vitro* was found to be strain specific, with only one of the three strains investigated producing significant quantities. A fragment of a gene encoding Pel was amplified from a number of strains, including those that did not produce Pel activity *in vitro*. However this gene was not detected in all *Xam* strains tested. *Pel* was found to be a single copy gene in *Xam* strain I56. From the evidence uncovered Pel does not appear to be a pathogenicity or virulence determinant in all strains of *Xam*.

Bacterial plant pathogens cannot enter the host directly and must rely on natural openings and wounds to initiate an infection. *Xam* was believed to enter cassava leaves through the numerous abaxial stomata. However the distribution of adaxial stomates along the mid rib of the leaf, where water collects and potentially where rain-spread bacteria would lie, indicated that they may provide a portal of entry for *Xam*. The density and distribution of adaxial stomates was investigated in terms of the relative resistance to *Xam* of several cassava cultivars. Glasshouse-grown cassava plants showed no correlation between the number or distribution of adaxial stomates and resistance to CBB. However, with cassava grown under field conditions a very susceptible cultivar (BEN) had significantly more adaxial stomates than a resistant cultivar (MNga2), indicating that stomatal density may play a role in resistance of some cassava cultivars to CBB.

In common with other plants of the *Euphorbiaceae* cassava produces copious quantities of latex on wounding. The role of latex in disease resistance is not known for any species, but the presence of antimicrobial proteins has often been reported. Cassava latex had

constitutive levels of β 1-3 glucanase, chitinase, lysozyme and protease activities. An EST library of latex revealed that 25% of non-redundant transcripts encoded proteins potentially involved in defence against biotic or abiotic stress, 29% encoded for proteins with a known function not involved in defence and 46% were genes of no known function.

Most plants produce a number of low molecular weight, antimicrobial compounds. These are either induced (phytoalexins) or constitutive (phytoanticipins). Bioassays of extracts from challenged and unchallenged cassava leaves revealed that the ethyl acetate soluble extract of unchallenged cassava leaves contained antimicrobial activity. The activity was detected at physiological concentrations and against a number of phytopathogenic bacteria (including *Xam*) and the fungal pathogen *Verticillium dahliae*. The activity was not increased by the hypersensitive response and was not extracted by a solvent of similar polarity to ethyl acetate (chloroform).

No cassava cultivar is completely resistant to *Xam.* Because of this limited expression of resistance, the tomato pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) was used as a more reliable inducer of cassava defences. A cDNA-AFLP (Amplified Fragment Length Polymorphism) based approach was used to uncover cassava genes with up-regulated or down-regulated expression in response to *Pst*. Around 1% of the cassava transcriptome was differentially regulated 24 h after inoculation with *Pst.* A number of novel cassava defence-related genes were isolated including a metalloprotease, a WRKY transcription factor and a calmodulin-like protein.

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He prodded the manioc with his fork (it tasted like half-set putty, marinated in Jeyes' Fluid for a week) and, contemplative, moved his tongue back and forth under his top lip, against his front teeth, where another piece had obviously stuck. He said, 'What the hell is this stuff?'

'Manioc. We lived on it in South America. Only there it usually came in wood-chipping and sawdust form. The Portuguese brought it over in the sixteenth century to feed to their slaves. The tuber's full of cyanide – you have to soak it, grate it, squeeze out the poison in a press of some kind, drive off any remaining moisture by roasting it in pans over a fire, and then you make the flour. Don't you like it?'

'Like it?' said Larry 'I could barf up my guts.'

Redmond O'Hanlon: Congo Journey

Chapter 1

Introduction

1.1 Cassava

Cassava (*Manihot esculenta* Cranz) is a crop cultivated throughout the tropics for its starch filled, tuberous roots. Approximately 700 million people depend on cassava as a staple foodstuff (de Mates and Vilarindes, 1998). In 2000 the cassava harvest was 171 million tons (FAO website), the seventh biggest harvest of any single food crop worldwide (table 1.1). Cassava can grow to a height of three metres, the stems and branches can reach eight cm in diameter (figure 1.1). Leaves are palmate and have between four and seven lobes and are attached to the stem by long petioles. Large swollen tuber-like roots develop underground (figure 1.2, figure 1.3). The starch filled roots are the major source of calories and the leaves are eaten as a vegetable in some African countries.

Starch grains found on milling stones in the Panamanian tropical forest have recently revealed that cassava was eaten 7000 years ago (Piperno *et al.,* 2000). 4600 year old pollen grains indicating cassava cultivation, have also been discovered in Mexico (Pope *et al.,* 2001). Genetic studies have shown that cassava is likely to have been domesticated from wild *M. esculenta* plants on the southern border of the Amazon basin and does not appear to be derived from several species as was originally thought (Olsen and Schaal, 1999). The Portuguese imported cassava from South America to the West Coast of Africa in the 16th century (Cock, 1985) as a cheap and easy to grow food for slaves. It spread to eastern Africa and India by the late 18th century. Cassava was brought to the Philippines from South America by the Spanish and arrived in Southeast Asia either *via* the Philippines or from India (Norman *et al.,* 1983).

Cassava is well suited to tropical regions; it has a high drought tolerance, is resilient to the poor soils which prevail in the tropics, exhibits resistance to most pests and diseases and has one of the highest yields of carbohydrate per hectare of any tropical crop (Cooke and Cock, 1989). Cassava requires relatively low maintenance with only minimal weeding necessary before harvest. Some cultivars are ready to harvest after six to nine months whilst others take as long as two years to develop reasonably sized swollen roots. Harvest may be delayed by up to two years with no real damage to the crop, thus making cassava an ideal buttress against starvation (Irvine, 1969). Cassava is suited to small farmers because of its ease of cultivation, delayed harvest and marketability. Sections of cassava stem cut from previous harvests and planted out sprout within seven days. The plants can then be left where they are to mature. Once the tuber-like roots have reached a harvestable size they can remain in the ground for two years with no significant loss in quality (Cock, 1985).The swollen root is an energy store for the plant, containing a large amount of

Figure 1.1 Cassava plantation

Figure 1.2 Cassava tuber like root
The starch filled tuberous root is the main source of calories from cassava

Crop	Production (tonnes)
Sugar Cane	1,281,767,380
Rice	597,154,664
Maize	589,355,356
Wheat	580,014,595
Potatoes	308,216,588
Sugar Beets	249,888,712
Cassava	**171,517,343**
Soybeans	161,042,126
Sweet Potatoes	141,069,941
Barley	132,896,783

Table 1.1 World Primary Crop Production 2000

Data taken from the FAO web site and shows the estimated harvest in tonnes for the ten greatest yielding crops for 2000.

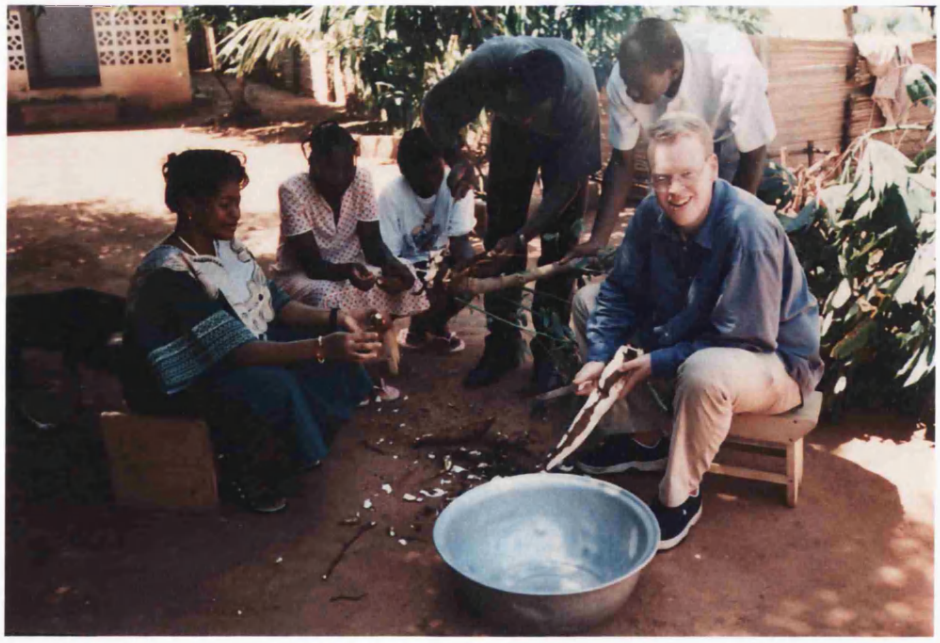


Figure 1.3 Preparation of cassava
The tuber-like root must first be peeled before soaking to remove cyanide

carbohydrate as starch but relatively little protein. Two thirds of cassava produced worldwide is consumed by humans as flour, breads, tapioca and meal (Cooke and Cock, 1989). Cassava also provides the raw material for processed products, ranging from laundry starch to industrial ethanol (Odigbon, 1983). Production of root crops, especially cassava, has been increasing faster than the population growth rate in some areas of Africa. In Nigeria and Ghana cassava production has doubled in ten years (Bokanga, 1998). When new technologies were introduced to encourage farmers to produce high quality cassava flour for the baking industry a significant increase in income was noted which encouraged farmers to try the new methods (Bokanga, 1998).

Cassava is not just a crop for small farmers, starch and starch derivatives such as dextrins, glucose and high fructose syrup are produced by most cassava growing countries (Cock, 1985). Whilst this is a cottage industry in Africa and South America, Thailand and Indonesia produce starch from cassava on a large scale. Thailand exports two million tons of starch from cassava annually (Sriroth *et al.,* 2000). The use of cassava as an industrial source of starch may provide the money needed to develop the crop. However several obstacles remain, including high production cost, starch loss and the environmental impact of the huge quantities of cyanide produced as a waste product (Sriroth *et al.,* 2000)

Cassava is resistant to many pests and diseases and this may be attributable to the presence of large amounts of cyanogenic glycosides (White *et al.,* 1998). Cyanogenic glycosides are involved in plant defence against insects (Schappert and Shore, 1999). This was recently demonstrated when the biosynthetic pathway for the synthesis of dhurrin, the cyanogenic glycoside from sorghum, was transferred into *Arabidopsis thaliana.* Plants accumulating dhurrin were resistant to attack from the flea beetle whereas untransformed plants were extremely susceptible (Tattersall *et al.*, 2001). Cyanogenic glycosides are low molecular weight compounds that consist of a glucose ring with a cyanohydrin (a cyanide group and a hydroxyl group attached to the same carbon atom) moiety attached. In cassava two cyanogenic glycosides are present, linamarin (about 90% of total cyanogen) and lotaustralin (about 10% of total cyanogen) in all tissues with the exception of the seeds. The highest levels of cyanogen are found in the leaves (five grams linamarin per kg fresh weight). Linamarin is localised in the vacuole and the linamarin hydrolysing enzyme linamarase is associated with the cell wall. Following tissue damage linamarin is exposed to linamarase and hydrolysed to yield glucose and acetone cyanohydrin. The release of acetone and HCN from the break-down of acetone cyanohydrin occurs spontaneously at pH greater than five or temperatures greater than 35°C and can be catalysed by the enzyme hydroxynitrile lyase (White *et al.,* 1998).

The preparation of cassava to remove most of the cyanide is a lengthy process. The tubers must first be milled or pounded to release linamarase, fermented for a few days to ensure the complete breakdown of linamarin and finally heated to drive off HCN and excess water (Cooke and Cock, 1989). Poorly prepared cassava contains residual cyanogen in the form of acetone cyanohydrin as the pH of the fermentation often drops below five (Tylleskar *et al.,* 1992). There are various problems associated with eating incorrectly prepared cassava tubers including hyperthyroidism, tropical ataxic neuropathy and konzo (Osuntokun, 1981, Cock, 1985, and Tylleskar *et al.,* 1992). These problems are exaggerated by a diet low in sulphur-containing amino acids, cysteine and methionine, as reduced sulphur compounds are essential for the detoxification of cyanide by rhodanase (Cock, 1985).

A major constraint on exploiting cassava as a cash crop is the rapid deterioration of the roots following harvest. Forty-eight h after harvest the majority of cassava cultivars are rendered inedible by an abiotic process termed post harvest physiological deterioration (PPD) (Beeching *et al.*, 1998). To avoid this problem cassava roots require rapid and elaborate processing to turn them into flour or dried chips (Cooke and Cock, 1989). PPD is dependent on the cultivar of cassava, the conditions of growth, conditions of storage and the damage that occurs to the root during harvest. The mechanism of PPD remains uncharacterised, however it is known to involve reactive oxygen species, cell death and a range of phenolic compounds (Beeching *et al.*, 1998).

Cassava is susceptible to a range of pests and diseases, which impose varying constraints on production. In total five bacterial diseases, eighteen fungal diseases and nine viral diseases are known to infect cassava (International Society for Molecular Plant Microbe Interactions – "Common Names for Plant Diseases" web site).

African cassava mosaic virus (ACMV) causes severe mosaic symptoms, chlorosis of areas of the leaf, reduction of leaf size and distortion of leaf growth in infected plants and had become endemic in some countries in Africa. It is spread by planting infected cuttings and by the insect vector *Bemisia* spp. (Whitefly) (Hahn *et al.,* 1979). Yield losses are a result of reduced photosynthetic capacity and can range from 20 to 95% in disease susceptible cultivars (Beck and Chant, 1958). A recent pandemic in the 1990's caused by the emergence of a hyper-virulent strain affected five countries and resulted in large economic losses and a threat to food supply (Legg and Thresh, 2000). Resistant varieties developed in the 1970s are still holding up and prevented the pandemic of the 1990s causing wide spread starvation (Legg and Thresh, 2000).

Two xanthomonads cause disease on cassava, *Xanthomonas axonopodis* pv. *manihotis (Xam)* causes Cassava Bacterial Blight (CBB) and *Xanthomonas campestris* pv. *cassavae* causes Cassava Bacterial Necrosis (CBN). CBB is the more serious disease and will be

discussed in depth later. Cassava anthracnose is a stem disease caused by *Colletotrichum* spp. resulting in canker, die back and defoliation, it has been reported to cause severe crop damage in areas of low soil fertility (Terry and Goodman, 1977).

Perhaps because of its high HCN content cassava is resistant to the majority of non-specific insect pests, however various insect pests do attack (Hanh *et al.,* 1979). Whitefly causes damage mainly as a vector of ACMV (Hanh *et al.,* 1979). The variegated grasshopper (*Zonocerus variegatus*) can defoliate cassava plants and reduce yield by up to 60% (Anonymous, 1974). The variegated grasshopper may also be a vector for the spread of CBB (K. Wydra personal communication). Cassava mealybug (*Phenacoccus manihot*) attacks the ends of cassava shoots and gains nutrients by sucking sap from the plants causing shoot stunting and a reduction in yield (Hahn *et al.,* 1979). The mealybug originated in Paraguay and once introduced to Africa, it was first reported in 1973, swept through cassava growing regions causing yield losses of up to 84% (Simarski, 1989). With no natural predators in Africa the mealybug became the major cassava pest until the introduction of the wasp *Apoanagyrus lopezi* as a biocontrol agent (Neuenschwander, 2001). In several countries the wasp cut losses due to mealybugs by more than half and the programme had a cost to benefit ratio of 1:149 (Simarski, 1989).

Another insect pest introduced from South America to Africa where it has had devastating effects is the green spider mite (*Mononychellus tanajoa*). The insect infects the underside of young leaves and feeds off sap. Heavy infection leads to chlorotic spots and eventually defoliation with resultant loss of yield (Hanh *et al.,* 1979). Again biocontrol has been attempted but with less success than with the mealybug (Simarski, 1989).

1.2 Cassava Bacterial Blight (CBB)

CBB is a disease of cassava caused by the bacteria *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). It is the most common biotic constraint on cassava production world-wide (Jorge *et al.,* 2000). CBB was first reported in South America in 1912 but did not reach Africa until 1970 (Maraite and Meyer, 1975). Losses from CBB can be as great as 100% as in Uganda in 1978 (Otim-Nape, 1980). The effect of CBB on a population reliant on cassava can be devastating; during epidemics in Zaire in the 1970's severe starvation was reported (Maraite and Meyer, 1975).

The reason for epidemics is often unknown; either a mutation within the bacterial population or a combination of conditions favourable to the disease, such as highly susceptible cultivars, impoverished soils and high rainfall, was blamed for the outbreak of CBB in Zaire in 1970 (Maraite and Meyer, 1975). Although CBB is under control in many

areas at present there is a real possibility that an epidemic may occur again (K.Kpémoua personal communication)

Initial infection or spread can occur through planting of infected cuttings, leaf damage or through stomates. It is possible that the pathogen undergoes an asymptomatic, epiphytic phase (Daniel and Boher, 1985). The symptoms of CBB are perhaps unique amongst bacterial diseases (Lozano, 1986). The first symptoms of CBB are the appearance of angular water-soaked lesions visible from three to seven days post infection on the leaf surface. Lesions spread and become chlorotic, eventually developing into brown necrotic lesions (figure 1.3). Ultimately lesions coalesce causing the death of the leaf that falls after a short time; the disease can then become systemic and cause vascular discoloration and disease of stem tissue, resulting in typical dieback symptoms (figure 1.4) (Maraite, 1993). The exudation of a sticky gum from leaf spots and cracks that develop on leaf surfaces and petioles is also characteristic of CBB (Lozano, 1986).

Much recent work has been focused on understanding the diversity of *Xam*. Variations within *Xam* populations have been detected at the biochemical, physiological and genetic level but no correlation between these characteristics and virulence have been reported (Maraite *et al.,* 1981, Van den Mooter *et al.,* 1987 and Verdier *et al.,* 1993). Ribosomal RFLPs indicate that *Xam* falls into five groups and diversity appears to be much greater in South American isolates than in African isolates. (Verdier *et al.,* 1993). All isolates from Africa fall into the same RFLP group whereas South American isolates are highly heterogeneous (Verdier *et al.,* 1993). This probably reflects that the centre of origin of cassava is South America. Detailed investigations into the population structure of *Xam* in different countries indicates that in South America *Xam* is locally adapted (Verdier *et al.,* 1997 and Verdier *et al.,* 1998a). Whilst some variation in African populations of *Xam* can be detected as a whole the population is very homologous, indicating a single point of origin relatively recently (Wydra *et al.*, 1998).

Control of CBB is effected by three main methods; improvement of cultural practices, varietal resistance and sanitation measures (Lozano, 1986). Often traditional methods of cassava cultivation such as planting material at the beginning of the rainy season, exchange of stem cuttings and planting cassava in the same area leads to an increased risk of CBB. Introduction of new planting regimes, the practice of crop rotation and appropriate sanitation methods should enable control of CBB and prevent epidemics (Lozano, 1989). However the most realistic method of control remains the use of resistant cultivars. Resistance to CBB has been developed through large scale breeding programmes in both Colombia (Colombian Institute for Tropical Agriculture, CIAT) and West Africa (International Institute for Tropical Agriculture, IITA). Resistance originates both from

Figure 1.4 Foliar symptoms

Typical chlorosis, necrosis and desiccation are evident in both artificially infected (left-hand panel) and naturally infected (right-hand panel) cassava leaves.

Figure 1.5 Dieback symptoms

Naturally infected cassava plant showing systemic infection and dieback.

cassava and from the wild relative of cassava, *Manihot glaziovii*. The resistance is thought to be polygenic, additively inherited and may be recessive to susceptibility (Hahn *et al.*, 1979). No completely resistant variety of cassava exists and all cultivars will show symptoms when inoculated with a high concentration of *Xam*. Resistance is expressed as a reduced level of disease development (Umemura and Kawano, 1983). Recent work has classified any cassava plant that has not shown wilting of more than two leaves 30 days after glasshouse inoculation as resistant, though many other symptoms will be present (Sanchez *et al.*, 1999, Restrepo *et al.*, 2000 and Jorge *et al.*, 2000). Resistance to CBB is dependent on climatic, edaphic and geographic factors (Jorge *et al.*, 2001). Development of CBB is highly dependent on temperature and rainfall with high humidity and warm temperatures leading to severe disease (Takatsu *et al.*, 1978). Cultivars that showed no symptoms in 1997 when rainfall was low (as a result of El Nino) were highly susceptible in 1998 when rainfall was at normal levels (Restrepo *et al.*, 2000). The survival of epiphytic populations of *Xam* is enhanced in warm, humid conditions and resistance is less likely when a high population of epiphytic bacteria is present (Daniels and Boher, 1985).

Whilst tropical regions are often perceived as having a single climate, cassava is grown under a variety of conditions. Cassava cultivars are adapted to the local environment, which has an influence on resistance to CBB (cultivars adapted to humid conditions are more likely to be resistant that those adapted to an arid environment). Cassava growing areas can be divided into seven edaphoclimatic zones (ECZ) depending on the climate and soil type. ECZ 1 = sub-humid tropics, ECZ 2=acid-soil savannas, ECZ 3 = humid tropical lowlands, ECZ 4 = mid-altitude tropics, ECZ 5 = high-altitude tropics, ECZ 6 = sub-tropics and ECZ 7 = semi-arid tropics (Anonymous, 1983). Field trials of a number of cassava cultivars indicate a variation in CBB resistance depending on ECZ and depending on which ECZ the cultivar was adapted to (Restrepo *et al.*, 2000). It is possible that variations in resistance are due to variations in pathogen population, as the cultivars in the study were inoculated with wild strains of *Xam* and it has been shown previously that diversity of *Xam* is related to ECZ (Verdier *et al.*, 1997).

Recently attempts have been made to define cassava resistance to CBB. The genetic diversity of cassava cultivars was assessed and correlated with resistance to CBB. Of the 93 varieties tested 49% were resistant to one or both strains of *Xam* used in the study, one from South America and one from Africa (Sanchez *et al.*, 1999). Half the resistant plants were only resistant to one strain of *Xam*, indicating that resistance to the two strains is independent (Sanchez *et al.*, 1999). Resistance was broadly spread around the germplasm and no correlation between genetic relatedness and resistance to CBB could be found (Sanchez *et al.*, 1999).

Quantitative trait loci (QTL) have been used in order to isolate areas of the cassava genome that are involved in resistance to CBB (Jorge *et al.,* 2000, Jorge *et al.,* 2001). A cross between two elite varieties of cassava previously used to create a genetic map (Fregene *et al.,* 1997) was used to generate 150 individuals for the trials. Initial greenhouse experiments revealed twelve different QTLs to be involved in resistance to five strains of *Xam* (Jorge *et al.,* 2000). Field trials later revealed eight QTLs involved in resistance to natural infection by *Xam* (Jorge *et al.,* 2001). One QTL is conserved between the greenhouse and field trials; it is suspected to be involved in the resistance phenotypes ingressed from *M. glaziovii* into the female parent of the cross (Jorge *et al.,* 2001). These experiments illustrate the intractability of CBB resistance to genetic dissection. Resistance is spread around the genome and appears to be independent for different strains of *Xam.*

The mechanisms of cassava resistance to CBB remain unknown. Ultrastructural investigation of infected plants found more xylem-associated defence responses in a resistant cultivar than in a susceptible cultivar (Kpémoua *et al.,* 1996). It has been suggested that resistance in only manifest in the xylem of infected plants (Boher *et al.,* 1996). However more recent work appears to show resistance in both the xylem and the leaves of cassava plants (Restrepo *et al.,* 2000a).

1.3 Bacterial Pathogenicity

1.3.1 Survival and Entry

Bacteria are often found living in close proximity to plants (Beattie and Lindow, 1999). The majority of these bacteria cause no apparent ill effect to the plant and some, such as nitrogen-fixing bacteria, are beneficial to the plant (Long, 2001). However the bacteria that do cause disease on plants are responsible for massive yield losses (Baker *et al.,* 1997).

Plant pathogenic bacteria often have an asymptotic epiphytic stage before causing disease (Beattie and Lindow, 1999). Bacteria land on leaves in an essentially random distribution. However they survive and multiply in specific, protected, areas notably at the base of the trichomes, around stomata and at the epidermal cell wall junctions (Beattie and Lindow, 1999). *Xam* has been reported to survive epiphytically without causing disease (Daniel and Boher, 1985). Epiphytic *Xam* have been localised round the stomata (Verdier *et al.,* 1990) although this study was conducted using *in vitro* plantlets, which are far removed from field grown cassava plants.

Pathogenic bacteria can also survive on seeds, dead plant material and on weed species growing in close proximity to host plants (Rudolph, 1993). *Xam* has been detected on the seeds of cassava plants (Persley, 1979a), surviving on weed species (Marcano and Trujillo, 1984), plant debris (Persley, 1979b) and in flood water (Joseph and Elango, 1991).

For epiphytic bacteria to cause disease they first have to enter the plant. Unlike fungal pathogens, some of which are able to enter the host actively (Knogge, 1996), foliar bacterial pathogens rely on passive entry into the plant (Wood, 1967). Chemotactic and aerotactic responses may help bacteria to locate points of entry (Rudolph, 1993). The main entry points are stomata, other natural openings and wounds (Alfano and Collmer, 1996). Insect vectors play a role in the transmission of some phytopathogenic bacteria, notably *Xylella fastidiosa* (Simpson *et al.,* 2000), and may occasionally play a role in the transmission of *Xam* (K. Wydra personal communication). Once in the apoplast the bacteria switch to pathogenic mode (Collmer, 1998).

Diseases caused by xanthomonads tend to start with the development of micro-colonies in the substomatal cavity (Rudolph, 1993). Rapid bacterial multiplication results in invasion of the leaf apoplastic space until the spread is halted by leaf veins, leading to the characteristic angular water soaking seen in xanthomonad diseases (Rudolph, 1993). In the case of *Xam* this initial stage also leads to infection of the xylem vessels and systemic disease (Maraite, 1993).

The intercellular spaces of plants contain sufficient nutrients for the initial stages of bacterial multiplication (two to three millimolar amino acids, three to six millimolar glucose and between 0.1 and 100 millimolar sucrose) (Novacky and Ullrich-Eberius, 1982). However for bacterial numbers to reach their maximal levels (xanthomonads multiply to between $1x10^8$ and $3x10^9$ cells per square cm of leaf tissue (Rudolph, 1993)) bacteria must be able to exploit the rich nutrient sources inside plant cells and avoid any defence responses the plant throws at them.

1.3.2 Manipulating plant cells

Recent studies have shown that phytopathogenic bacteria are probably able to translocate effector proteins into the plant cell (Lahaye and Bonas, 2001). The ability to secrete effector proteins is essential to pathogenicity for a number of bacteria and is encoded by a cluster of hypersensitive response and pathogenicity (*hrp*) genes (Lindgren, 1997). A number of these genes encode a type III secretion system that transports proteins across the bacterial membrane (Grant and Mansfield, 1999). Type III secretion systems are conserved in both plant and animal pathogenic bacteria (Staskawicz *et al.,* 2001) and are often referred to as a "molecular syringe".

The Hrp pilus is constructed from pilins encoded in the *hrp* gene cluster (Collmer, 1998) and is required for secretion and delivery of various effector proteins to the plant cell (Grant and Mansfield, 1999). The pilus can cross the cell wall barrier and effector proteins are either injected through the pilus into the plant cell or guided along the pilus to the plant

cell (Staskawicz *et al.,* 2001). Although the type III secretion system has not been reported in *Xam* it has been reported for a number of other *Xanthomonas* pathovars and it is likely to be present in *Xam.*

Effector proteins are often referred to as Avirulence (Avr) proteins because of their HR-eliciting activity, however in keeping with current literature, I will refer to them as effector proteins. Many effector proteins are involved in virulence on susceptible plants (Kjemtrup *et al.,* 2000; White *et al.,* 2000). It appears that bacterial effector proteins originally evolved as virulence factors and were then targeted by plants as suitable triggers for initiating defence responses hence their role as avirulence determinants (Lahaye and Bonas, 2001). The "guard model" of Avr/R protein interaction, suggesting that the Avr protein must first bind to a pathogenicity target molecule before recognition by the plant Resistance (R) protein, adds credence to the theory that Avr proteins have a role other than eliciting the defence response (Dangl and Jones, 2001).

All bacterial effector proteins examined to date are secreted *via* the *hrp* encoded secretion system (Lahaye and Bonas, 2001). A number of effector proteins from xanthomonads have been isolated (Daniels and Leach, 1993). Whilst the biochemical function for most effector molecules is unknown three families of effector proteins that have been characterised in some detail.

AvrBs2 was first isolated from *X c.* pv. *vesicatoria* and has also been found in a number of other *X. campestris* pathovars (Kearney and Staskawicz, 1990). AvrBs2 has homology with enzymes that synthesise or hydrolyse phosphodiester bonds though the role of this homology in virulence function is unknown (Swords *et al.*, 1996). AvrBs2 is required by the pathogen for full virulence and is detected by the Bs2 protein in resistant cultivars of pepper (Tai *et al.,* 1999). The *Bs2* from pepper can be transferred into tomato to give resistance to *X. c.* pv. *vesicatoria* in tomato (Tai *et al.,* 1999). Recently (as part of this study) *Xam* was tested for AvrBs2 activity, however no activity was detected (B. Staskawicz, personal communication).

The AvrBs3 family of proteins was also first detected in *X. c.* pv. *vesicatoria* and is found in a large number of xanthomonads (Vivian and Arnold, 2000). AvrBs3 homologues are probably present in *Xam* (Verdier *et al.,* 1998a). Some xanthomonads, such as *X. oryzae* pv. *oryzae*, contain several members of the *avrBs3* family (Bai *et al.*, 2000). AvrBs3-like proteins contain two eukaryotic structures, a functional nuclear localisation signal and an acidic transcriptional activation domain (Szurek *et al.*, 2001). The nuclear localisation signal interacts with importin α, which traffics proteins from the cytoplasm to the nucleus (Szurek *et al.*, 2001). This appears to be the bacteria hijacking host machinery to deliver

effector proteins to their site of action. The transcriptional activation domain is then hypothesised to function as a transcription factor, changing the host's transcriptome to the benefit of the pathogen (Lahaye and Bonas, 2001).

Xam appears to contain several members of the *avrBs3* family (Verdier *et al.,* 1997). A plasmid-borne pathogenicity gene, designated *pthB*, encodes a protein with a high degree of similarity to effector proteins from the AvrBs3 family (Verdier *et al.,* 1998a). *pthB* is reported to be absent from non-pathogenic strains of *Xam* (Verdier *et al.,* 1998a). Between one and nine copies of the gene appears in *Xam* strains, located both on the chromosome and on plasmids (Restrepo and Verdier, 1997). The high copy number of *pthB* is similar to the copy number of *avrBs3* family genes in a number of xanthomonads (Layahe and Bonas, 2001). Whilst *pthB* has been used in PCR (Verdier *et al.,* 1998b) and hybridisation based detection methods (Verdier and Mosquera, 1999) for *Xam* no biochemical data showing its role in pathogenicity has been reported to date.

The YopJ family of proteins has been found in a number of mammalian pathogens as well as plant pathogens and plant symbionts (Staskawicz *et al.*, 2001). They are proposed to be cysteine proteases, and mutations in the conserved protease motif inactivates the proteins (Staskawicz *et al.*, 2001). The best-studied members of this family are YopJ and YopP from *Yersinia* spp. They promote apoptosis of macrophages (but not other cell types) and inhibit the production of cytokines thus affecting the immune response (Cornelis and Denecker, 2001). The role of YopJ homologues from xanthomonads is not yet clear, but interference with host resistance responses is a possibility (Lahaye and Bonas, 2001). As the roles of more effector proteins are uncovered it will be possible to see how bacteria manipulate plants to their advantage.

1.3.3 Damaging plant cells

In addition to the elegant manipulation of plant cells achieved by hrp secreted effector proteins, phytopathogenic bacteria have less subtle methods of gaining nutrients from the host. These virulence factors are not targeted against individual plant cell. Secreted virulence factors include plant cell wall-degrading enzymes, toxins, siderophores, hormones and signalling molecules (Salmond, 1994). Some of these have been linked with the virulence of xanthomonads.

The plant cell wall is a rich source of nutrients and many plant pathogens make one or more enzymes capable of degrading the polymers in plant cell walls (Walton, 1994). Ultrastructural studies of *Xam* infected cassava plants reveal degradation of cassava cell walls and release of pectin fragments, suggesting a role for cell wall degrading enzymes in *Xam* pathogenicity (Boher *et al.,* 1995). In phytopathogenic bacteria cell wall-degrading

enzymes are secreted by the type II secretion pathway (Alfano and Collmer, 1996). The type II secretion pathway is a two step process that first translocates proteins from the cytoplasm to the periplasm and then across the outer membrane (Salmond, 1994). Mutants in *X. c.* pv. *campestris* (Hu *et al.*, 1992) and *X. oryzae* pv. *oryzae* (Ray *et al.*, 2000) type II secretion pathways accumulate extracellular enzymes in the cytoplasm and periplasm and have reduced virulence when compared to wild-type bacteria. The role of individual enzymes secreted by the xanthomonas type II secretion pathway is unclear but has been the subject of much research.

The degradation of pectin involves several enzymes that often act synergistically (Collmer and Keen, 1986). Production of pectolytic enzymes is common among xanthomonads though by no means ubiquitous (Dye, 1960). *Xam* produces pectin methylesterase (PME) which removes methyl groups from the pectin chain (Ikotun, 1984). *Xam* has also been reported to macerate potato slices, a standard test for production of pectin degrading enzymes (Dos Santos and Dianese, 1985). Some strains of *Xam* produced large amounts of pectate lyase (Pel) when grown on cassava cell walls (Deshappriya, 1992). Pel cleaves a demethylated pectin chain and is a pathogenicity factor in soft-rot bacteria (Collmer and Keen, 1986). The most intensively studied bacterial Pels are from the soft-rotting *Erwinia* spp. which secrete a number of different Pel isoenzymes (Barras *et al.,* 1994). Pel enzymes from *Erwinia* are divided into four groups based on sequence homology; the extracellular PelADE, extracellular PelBC, periplasmic Pels and Pel3 from *E. carotovora* (Heffron *et al.*, 1995).

Pels from a number of xanthomonads have been studied in detail, including *X. c.* pv. *campestris* (Dow *et al.,* 1989), *X. c.* pv. *vesicatoria* (Beaulieu *et al.*, 1991) and *X. c.* pv. *malvacearum* (Liao *et al.*, 1996). A number of xanthomonads have also been shown to carry a *pel* gene and produce Pel *in vitro* (Liao, *et al.*, 1996). *Xam* appears to produce one isozyme of Pel (Deshappriya, 1992), which is similar to most xanthomonads studied (Liao *et al.*, 1996). However *X. c.* pv. *campestris* produces three isozymes (Dow *et al.*, 1989). The role of Pels in pathogenicity of xanthomonads has yet to be fully established although they appear not to be required, as many pathogenic strains do not produce Pel. Production of Pel is highly variable within *Xam* strains, though does not appear to correlate with virulence (Deshappriya, 1992). Both pectolytic and non-pectolytic strains of *X. c.* pv. *vesicatoria* were fully pathogenic (Beaulieu *et al.*, 1991) as were strains of *X. c.* pv. *campestris* with an inactive copy of one *pel* gene (Dow *et al.,* 1989). Also four strains of *X. c.* pv. *malvacearum* with different levels of Pel activity *in vitro* were found to cause similar disease symptoms (Liao *et al.,* 1996). Pel is known to induce cell death in a number of plant cells (Collmer and Keen, 1986) and was the only factor identified from *Xam* which

killed cassava suspension cells (Deshappriya, 1992). Pel may be involved in survival of xanthomonads outside the host as a number xanthomonads, including *Xam*, that produce very high levels of pectolytic enzymes cause soft-rot like symptoms in potato slices (Liao *et al.*, 1996 and Dianese and Dos Santos, 1985). This may indicate a role for Pel in opportunistic survival of xanthomonads (Liao *et al.*, 1996) or in survival on dead plant tissue.

In some systems the action of pathogen pectolytic enzymes release cell wall oligosaccharides that act as elicitors of a plant defence response (Ryan and Farmer, 1991). Indeed, transgenic potato tubers producing pectate lyase from *Erwinia carotovora* showed increased resistance to that pathogen (Wegener *et al.,* 1996).

Another major plant cell wall component is cellulose, degradation of which is mediated by cellulase and has been reported for a large number of xanthomonads (Rudolph, 1993). Endoglucanase is the major extracellular enzyme produced by *X. c.* pv. *campestris* when grown in a rich medium (Gough *et al.,* 1988). However a mutant that lacked cellulase activity was as virulent as wild type bacteria (Gough *et al.,* 1988). Ultrastructural studies indicate that cellulose degradation occurs in cassava cells infected with *Xam* but the cause of this was not determined conclusively (Boher *et al.,* 1995).

In addition to polysaccharides the plant cell wall contains a number of structural proteins (Showalter, 1993). Of these proteins two classes of glycoproteins are implicated in plant defence against pathogens, the hydroxyproline-rich glycoproteins (HRGPs) and proline-rich proteins (PRPs) (Bowles, 1990). Extracellular proteases have been detected in a number of xanthomonads. Mutants with defective protease activities in *X. c.* pv. *malvacearum* (Gholson *et al.,* 1989), *X. c.* pv. *campestris* (Dow *et al.,* 1990) and *X. oryzae* pv. *oryzae* (Xu and Gonzalez, 1989) showed reduced virulence. Whilst most pathogen proteases are detected using model substrates such as gelatine and casein, a metalloprotease was recently isolated from *X. c.* pv. *campestris* that degraded specific HRGPs and PRPs from a number of plants (Dow *et al.,* 1998). This is the only protease (of four so far discovered in *X. c.* pv. *campestris*) that can degrade PRPs from turnip, which have previously been shown to be induced by wounding and infection (Davies *et al.*, 1997). This may indicate that this particular protease is involved in pathogenicity (Dow *et al.,* 1998).

Production of extracellular enzymes in xanthomonads is tightly regulated (Dow and Daniels, 1994), probably because of the varying environmental and physiological conditions xanthomonads encounter during the disease cycle. For example *Xam* will produce Pel when grown in a minimal medium with plant cell walls as the sole carbon source but not in a complex medium (Deshappriya, 1992).

In *X. c.* pv. *campestris* a number of regulatory genes have been isolated that control the production of both extracellular enzymes and extracellular polysaccharide (EPS). Mutations in *rpf* (regulation of pathogenicity factors) genes lead to the down regulation of extracellular enzymes and EPS production leading to a corresponding loss of pathogenicity (Dow and Daniels 1994). At least nine genes (*rpfA-I*) are present in the *rpf* cluster and the function of some of these genes has been assigned (Dow *et al.*, 2000a). *rpfA* encodes an aconitase that may be involved in regulation of gene expression in response to iron levels (Wilson *et al.*, 1998). *rpfF* and *rpfB* are involved in mediating extracellular enzyme production by a low molecular weight diffusible signal factor (DSF) (Barber *et al.*, 1997). Interestingly xanthomonads do not tend to produce *N*-acyl homoserine lactones (*N*-AHLs), the most common signalling molecule found in gram negative bacteria and the mediator of quorum sensing (Cha *et al.*, 1998). It is possible that xanthomonads use DSF instead of *N*-AHLs in cell-cell signalling (Slater *et al.*, 2000). DSF probably acts on a signal transduction system encoded by *rpfC, rpfG* and *rpfH* (Tang *et al.*, 1991, Slater *et al.*, 2000). A *rpfC* homologue has been detected in *X. oryzae* pv. *oryzae* and mutants were less virulent and produced less EPS that wild type bacteria (Tang *et al.*, 1996). Many of the *rpf* genes have homologues in *Xylella fastidiosa* (da Silva *et al.*, 2001)

Extracellular enzyme and EPS production in *X. c.* pv. *campestris* also appear to be regulated by a gene similar to catabolite activation factor. Mutations in this gene result in much reduced pathogenicity (De Crecy-Lagard *et al.,* 1990).

Whilst not well characterised in Xanthomonads the production of toxins which target the host cells is a feature of many plant pathogenic bacteria (notably *Pseudomonas syringae* pathovars) (Alfano and Collmer, 1996). The *Pseudomonas* toxins are mostly small peptides and show no host specificity (Gross, 1991). For example *P. syringae* pv. *syringae* produces syringomycin and syringopeptin, which are cyclic peptides that induce necrosis by forming pores in plant plasma membranes and *P. s.* pv. *tabaci* produces tabtoxin a β-lactam-containing dipeptide that inhibits glutamine synthase and causes chlorosis (Alfano and Collmer, 1996). The role of these toxins *in planta* is unknown and it had been suggested that they may be involved in reducing microbial competition during the epiphytic phase of the bacterial life cycle (Gross, 1991). However coronatine, produced by *P. s.* pv. *tomato*, may suppress defence gene activation by mimicking plant signal molecules (Mittal and Davis, 1995) and syringomycin induces the opening of calcium channels that may disrupt calcium signalling in plants (Hutchison *et al.,* 1995).

It had been suggested that a carboxylic acid toxin (3(methylthio) propionic acid) produced by *Xam* was responsible for the blight symptoms seen in CBB (Perreaux *et al.,* 1982). However this "toxin" only produced leaf symptoms at unrealistically high concentrations

and neutralisation of the acid removed all toxic effects (R. Day and R. Cooper personal communication)

1.3.4 Extracellular Polysaccharides

Plant pathogenic bacteria often produce large quantities of extracellular polysaccharide (EPS) and lipopolysaccharide (LPS) in culture and *in planta* (Denny, 1995). EPS is secreted from the bacteria as slime or capsular material and different species of bacteria produce different forms. LPS is produced by all gram-negative bacteria and is associated with the outer membrane.

The EPS produced by xanthomonads is termed xanthan and has uses both as a thickening agent in food production and as a suspension agent, flocculent, viscosity control agent, stabiliser and gelling agent in various industrial processes (Sutherland, 1993). The estimated annual production is at least 20,000 tonnes (Sutherland, 1993).

Many roles have been suggested for xanthan *in planta* mainly involved in protecting bacteria from environmental stresses (Denny, 1995). Early work showed that encasing *X. c.* pv. *phaseoli* in xanthan increased the thermal death point and tolerance to UV light (Leach *et al.,* 1957). Xanthan may protect xanthomonads from desiccation, concentrate minerals and nutrients, reduce contact with toxic compounds (either from plants or other micro-organisms), enhance the attachment to surfaces, enhance spread through the plant and sustain water-soaking of intercellular spaces (Denny, 1995). Xanthan may also be involved in the production of biofilms, which are crucial to the pathogenicity of some mammalian pathogens (Stickler, 1999). Xanthan-deficient mutants of *X. campestris* often have reduced virulence when compared to wild type bacteria (Corey and Starr, 1955, Sutton and Williams, 1970, Daniels *et al.,* 1984 and Shaw *et al.,* 1988). However unless the mutation is clearly defined and not pleiotropic (as is often the case with mutations in regulatory genes) no conclusions on the role of EPS in pathogenicity can be drawn.

Xanthan production is initiated *in planta* 1-2 h after inoculation of bean with *X. campestris* pv. *vesicatoria* (Rudolph *et al.,* 1989). No difference was seen between the quantity of xanthan produced in resistant and susceptible bean cultivars up to 24 h after inoculation (Brown *et al.,* 1993). Xanthan from *Xam* has been localised in the intercellular spaces of cassava plants (Boher *et al.,* 1995; Boher *et al.,* 1997). Immuno-detection showed xanthan from *Xam* filling the intercellular spaces and often present in advance of invading bacteria (Boher *et al.,* 1995). Xanthan was also localised close to degraded cell walls. The authors suggest either that xanthan provides a matrix for cell wall degrading enzymes to reach the cell wall (Boher *et al.*, 1995) or that xanthan binds to plant polymers to form a gel "that

may have a striking effect on plant colonisation by xanthomonads" (Boher *et al.,* 1997). Quite what the striking effect is was not revealed.

Because of the industrial importance of xanthan the structure and biosynthetic pathway have been studied in great detail. Xanthan is a polymer composed of many pentasaccharide repeat units. The unit is formed by the addition of monosaccharides from sugar nucleotides to a C_{55} isoprenoid lipid acceptor molecule (Sutherland, 1993). These units are then polymerised to give a β-1,4-glucan (cellulose like) backbone with a trisaccharide side chain of mannose-glucuronic acid-mannose on every alternate glucose molecule (figure 1.5) (Jannson *et al.*, 1975). The first mannose unit of the side chain is acetylated and the end mannose unit may be pyruvated (figure 1.5).

A cluster of 12 genes are involved in the biosynthesis of xanthan (Harding *et al.,* 1987; Katzen *et al.,* 1998). These genes are termed *gum* genes and are named *gumB* - *gumM* in the order they appear on the *X. c.* pv. *campestris* genome (Katzen *et al.,* 1998). GumD, GumM, GumH, GumK and GumI are involved in the formation of the pentasaccharide repeat unit, GumF and GumG acetylate manose, GumL pyruvates mannose and GumB, GumC and GumE are involved in polymerisation of the pentasaccharide unit and export of xanthan (figure 1.6). Homologues of a number of gum genes have been detected in the genome sequence of *Xylella fastidiosa* (da Silva *et al.,* 2001). It is speculated that *Xylella fastidiosa* produces an EPS similar to xanthan, possibly involved in pathogenicity (da Silva *et al.,* 2001).

The GumD protein catalyses the first step of xanthan production and is responsible for the addition of the first glucose molecule to the polyprenol (Katzen *et al.,* 1998 and figure 1.6). Mutants of *X. c.* pv. *campestris* with no *gumD* produce no xanthan (Katzen *et al.,* 1998). When any other of the gum biosynthetic genes is mutated it either leads to the production of limited quantities of EPS or the build up of toxic intermediates (Katzen *et al.,* 1998).

The virulence of a number of *gum* mutants has been assessed. *X. c.* pv. *campestris* with no functional *gumD* showed reduced virulence when compared to wild-type bacteria (Katzen *et al.,* 1998; Chou *et al.,* 1997). An *X. oryzae* pv. *oryzae* mutant producing very little EPS was defective both in causing symptoms on rice plants and in spread throughout the plant (Dharmapuri and Sonti, 1999). This mutant was produced by transposon mutagenesis and was found to be defective in a gene similar to *gumG* (Dharmapuri and Sonti, 1999). This is surprising as *gumG* in *X. c.* pv. *campestris* is involved in the acetylation of xanthan monomers and mutants in *gumG* still produce high levels of EPS (Katzen *et al.,* 1998). However the transposon insertion may have caused effects on the expression of

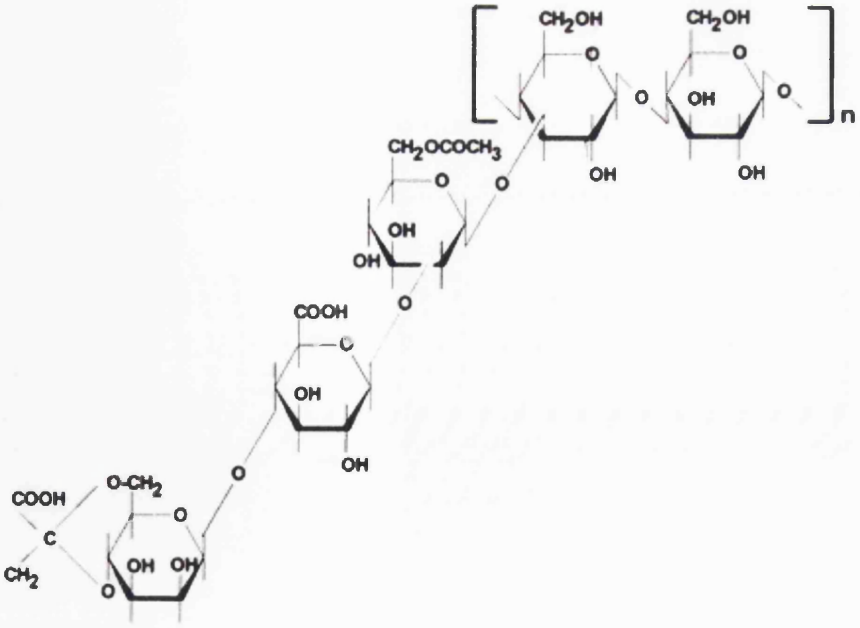


Figure 1.6 The structure of xanthan from *X. campestris* pv. *campestris*

The above structure is of the pentameric repeating unit of xanthan. The final structure is essentially a cellulose backbone with trisaccharides attached to every other glucose. Figure taken from Katzen *et al.* (1998).

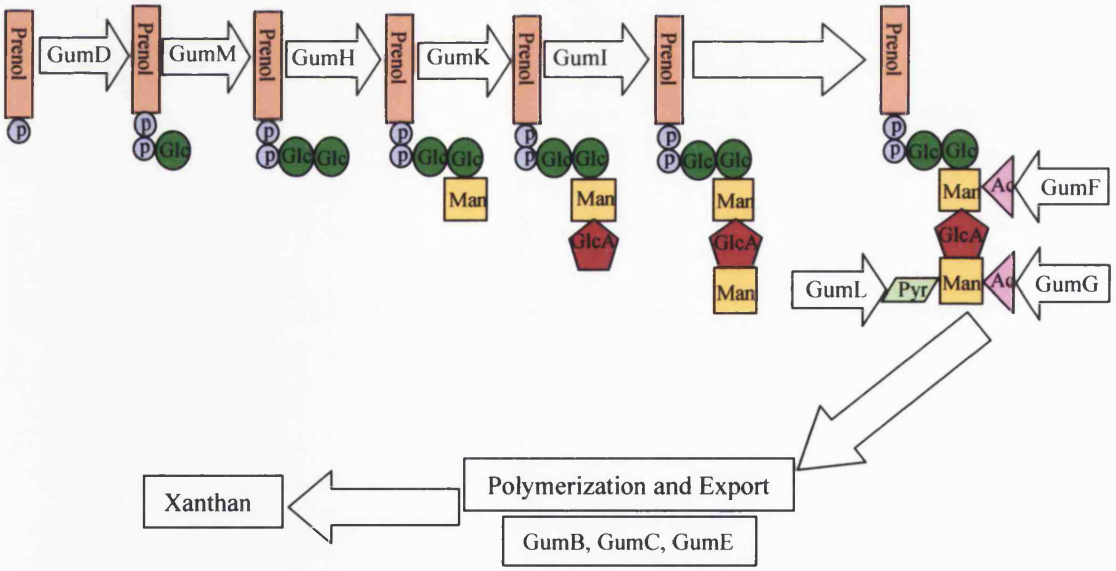


Figure 1.7 Biosynthesis of Xanthan in *Xanthomonas campestris* pv. *campestris*. A C_{55} isoprenol lipid molecule (Prenol) accepts a number of monosaccharides in order to create the oligosaccharide repeat unit of xanthan. Acyl (Ac) and pyruvate (Pyr) adornments are added to the finished repeat unit before they are polymerised in xanthan gum and exported out of the bacterial cell. Glc = glucose, Man = Mannose and GlcA = glucuronic acid. Enzymes encoded by the *gum* gene cluster are in white arrows. Figure adapted from Katzen *et al.* (1998).

downstream genes as the *gum* genes are mainly expressed as an operon (Dharmapuri and Sonti 1999).

Regulation of EPS production is complex and linked to a number of factors. Kamoun and Kado (1990) describe how various *X. campestris* pathovars exhibit phenotypic switching between wild type (EPS producing and non chemotactic) and swarmer type (EPS minus and chemotactic) on swarm-plates. The swarmer type *X. c.* pv. *campestris* were less virulent than wild type and would only revert to wild type *in planta*. The authors suggested that as both EPS production and chemotaxis are energy expensive xanthomonads have developed a way of regulating them for use in the most suitable environment (Kamoun and Kado, 1990).

EPS production is also co-regulated with extracellular enzymes by the *rpf* group of genes (Dow and Daniels, 1994, see above for more details) and a *X. c.* pv. *campestris* homologue of catabolite activation factor (De Crecy-Lagard *et al.,* 1990).

Another small diffusible factor (DF) in addition to DSF (see above) has been implicated in regulating EPS production (Poplawsky *et al.,* 1998). DF appears to regulate EPS and pigment (xanthomonadin) production in *X. c.* pv. *campestris* but does not have any effect on extracellular enzyme production (Poplawsky *et al.*, 1998). Mutants that do not produce DF do not produce EPS or xanthomonadin *in vitro* and have much reduced epiphytic survival and host infection though hydathodes (Poplawsky and Chun, 1998). However when inoculated into wounds the mutants are as virulent as wild type bacteria (Poplawsky and Chum, 1998). This indicates that EPS production is regulated by environmental conditions.

All gram-negative bacteria produce LPS which is located on the outer plasma membrane. LPS is speculated to restrict the permeability of the outer membrane and, because of its localisation on the cell surface, interact with eukaryotic cell surfaces (Dow *et al.*, 2000b). LPS activates immune responses in both mammalian and insect cells (Qureshi *et al.*, 1999). Pre-treating plants with purified LPS, from any source, prevents the hypersensitive response when plants are infected with avirulent bacteria (Dow *et al.,* 2000b). It is thought that pre-treatment sensitises the plant to pathogens resulting in inhibition of bacterial growth so the HR, which requires active bacterial metabolism, is not triggered (Dow *et al.,* 2000b). *X. c.* pv. *campestris* strains that produce defective forms of LPS are less pathogenic and more susceptible to antimicrobial agents (Dow *et al.,* 1995).

LPS from *Xam* has been localised *in planta* using immunolabeling (Boher *et al.,* 1997). Early in infection LPS was located on the bacterial outer membrane and in areas of the plant middle lamellae close to *Xam* cells. However LPS was localised to areas where the plant cell wall was heavily degraded during later stages of infection (Boher *et al.*, 1997).

The authors hypothesised that LPS is somehow involved in altering plant polysaccharides and is translocated to the cell wall "*via* the xanthan fibrillar network" (Boher *et al.,* 1997). They admit that as the binding specificity of their LPS antibody has not been defined and as galacturonic acid is present in the water soluble LPS from *X. campestris* (Volk, 1966) cross reactivity between degraded cell walls and LPS may explain the labelling pattern (Boher *et al.,* 1997).

I think it unlikely that the interactions between LPS (or EPS) and plant cell walls has any significant relevance for pathogenicity and feel the interactions observed by Boher and co-workers are artefactual. Increasing evidence suggests that plant pathogenic bacteria have evolved mechanisms to limit the defence responses initiated by LPS (Dow *et al.*, 2000b) and it seems unlikely that pathogens would target LPS to plant cells. Especially when the function (alteration of the host cell wall) would be much better achieved with a specific enzyme that would not trigger host defences.

1.4 Plant defences against pathogens

Plants lack two of the main defence mechanisms used by animals; the ability to move away from predators and a circulatory system within which an immune system can evolve. Plants have evolved mechanisms to limit the loss of energy to pathogens to such an extent that most plants are resistant to most pathogens (Dangl and Jones, 2001). Indeed the co-evolution between plants and their natural enemies is believed to have generated much of the Earth's biodiversity (Rausher, 2001). Defences can be divided into two areas: host resistance and non-host resistance. Non-host resistance is where a whole plant species is resistant to a specific pathogen and is the most common form of resistance in plants (Heath, 2000). Host resistance is generally narrowly defined as the result of the interactions between host *R* (resistance) genes and pathogen *avr* (avirulence) genes (Dangl and Jones, 2001). As cassava resistance to bacterial blight is not under single gene control (Hahn *et al.,* 1979), I will refer to host resistance as the varying resistance phenotypes of a plant species to a specific pathogen. The prevention of pathogen spread occurring in both host and non-host resistance appears to be mediated by similar mechanisms. These can be divided into preformed defences and induced defences.

1.4.1 Preformed defences

The most efficient way to stop infection is for a plant to prevent pathogen entry. To this aim plant leaves are covered in cuticle, a hydrophobic layer providing a waxy "skin". Cuticle is made of the water-insoluble polymer cutin in a mixture of hydrophobic compounds collectively called wax. This wax is also found in crystalline form on the

surface of plants (Kolattukudy and Köller, 1983). The cuticle provides both a structural and a chemical barrier to pathogen attack (Crute *et al.,* 1985) and fungal pathogens have, in turn, evolved cutinases and/or use high pressure to breach the cuticle.

Bacterial pathogens have no means of actively entering the host and rely on passive entry into the plant (Wood, 1967). The main entry points are *via* stomates, other natural openings and wounds (Alfano and Collmer, 1996). Several stomatal features may play a role in disease resistance; size, opening characteristics, shape, structure, density and distribution of stomates have all been suggested as being important in different systems.

McLean related the resistance of citrus leaves to *Pseudomonas citri* to stomatal shape and size in 1921. The mandarin orange is suggested to be resistant to the disease due to very narrow stomatal openings and that the stomates are raised above the plane of the leaf, features absent in the susceptible grapefruit (McLean, 1921). When bacteria are inoculated through wounds or forced into the sub stomatal cavity under pressure, both species are equally susceptible (McLean, 1921). This is thought to be the only example of stomatal structure alone controlling resistance (Crute *et al.,* 1985). A study of eight *Lycopersicon* species revealed a relationship between resistance to bacterial leaf spot caused by *Xanthomonas campestris* pv. *vesicatoria* and frequency of stomata, stomatal size and some morphological leaf characteristics. Significant correlation was found between the stomatal frequency in both adaxial and abaxial leaf surfaces and the number of bacterial lesions per leaf. The stomatal width was also correlated with the number of bacterial lesions per leaf. Electron microscopy revealed those morphological features, such as raised stomatal complexes and hydrophobic waxes may also play a role in resistance (Ramos *et al.,* 1992).

Resistance of hyacinths to yellow disease caused by *X. campestris* pv. *hyacinthi* has been suggested to be in part due to differences in stomatal size and distribution. Tetraploid cultivars with a few, large stomata tended to be more susceptible than diploid plants with many small stomata (Van Tuyl, 1982). However as *X. c.* pv. *hyacinthi* is known to enter hyacinths through the hydathodes a role for stomatal size in determine resistance is unclear (Huang, 1986).

In addition to morphological preformed defences plants also utilise a range of chemicals with antimicrobial properties (Heath, 2000). Peptides (Broekaert *et al.,* 1995) and proteins (Bowles, 1990) have both been characterised as preformed antimicrobial chemicals but by far the most researched class is that of low molecular weight compounds.

Low molecular weight, constitutively synthesised compounds found in plants with antimicrobial properties are termed phytoanticipins (VanEtten *et al.,* 1994). Both compounds that exist in plants in their biologically active form and compounds which

occur as biologically inactive precursors and are activated in response to tissue damage or pathogen attack are included in this grouping (Osbourn, 1996). However the term may be misleading as a compound (for example saukuranetin) that is produced in response to pathogen attack in one species (in this case rice) may be constitutively present in another (blackcurrant) (Dixon, 2001).

A large number of constitutive plant compounds with antifungal activity have been reported, including phenols, unsaturated lactones, sulphur compounds, cyanogenic glucosides and glucosinolates (Osbourn, 1996). Resistance of onion to *Colletotrichum circinans* has been related to the phenolic compounds catechol and protochatechuic acid. These compounds are present in the outer scales of the disease resistant red onion, and absent in the outer scales of disease susceptible white onion (Walker and Stahmann, 1955). The best characterised model for host resistance and preformed antifungal agent is in the interaction between oat and the "take all fungus" *Gaeumannomyces graminis* var *tritici* (Osbourn, 1996). Though this fungus causes disease in wheat it is unable to infect oat, because it is susceptible to the saponin avenacin which is present in oat roots (Osbourn, 1996). *G. graminis* var *avenae* is able to infect oats because it contains an enzyme, avenacinase, which detoxifies avenacin. When *G. g.*var *tritici* is transformed to express avenacinase it is able to infect oats (Osbourn, 1996). Saponin deficient (*sad*) mutants of oat are less resistant to *G. g. var tritici* and avenacin content and disease resistance correlate in a segregating population (Papadopoilou *et al.,* 1999).

In contrast to the well-characterised roles of phytoanticipins in fungal diseases there are few reports of preformed antibacterial compounds involved in plant diseases. Strains of *Erwinia chrysanthemi* pathogenic on maize are less sensitive to the antibacterial cyclic hydroxamate found in some maize lines than non-pathogenic strains (Lacey *et al.*, 1969). However there was no evidence that maize lines that did not produce hydroxamate were any more susceptible to *E. chrysanthemi* infection (Lacey *et al.,* 1969). Resistance of tomato lines to *Ralstonia solanacearum* has been related to production of the saponin α tomatine (Mohanakumaran *et al.*, 1969). The phenolic glucoside arbutin has been implicated in resistance of pears to fire blight caused by *E. amylovora.* Oxidation of arbutin results in the production of an antibacterial hydroquinone. Fire blight resistant cultivars of pear had higher levels of arbutin than susceptible cultivars and arbutin was concentrated in the tissues of pear most resistant to infection (Hildebrand *et al.,* 1969). The unsaturated lactone tuliposides from tulips are both anti-fungal and anti-bacterial (Mansfield, 1983).

Cassava is well known for production of the cyanogenic glycosides linamarin and lotaustralin which release hydrogen cyanide in response to tissue damage (see above).

Whilst the role of cyanogenic glycosides in defence against herbivory is well defined (Schappert and Shore, 1999) the role of cyanogenic glycosides in plant disease resistance has yet to be fully understood. It has been suggested that all pathogens of cyanogenic plants must contain either enzymes to detoxify the cyanide (cyanide hydratase) or possess cyanide-resistant respiration (VanEtten *et al.,* 1994). In the only detailed study to date mutants of *Gloeocercospora sorghi* without cyanide hydratase activity were still fully pathogenic on the cyanogenic host, sorghum (VanEtten *et al.,* 1995). The role of cyanogenic glycosides in resistance to CBB or any other disease has not been determined.

Along with several thousand other plant species cassava produces latex. Latex is the cytoplasm of highly specialised cells called laticifers (Martin, 1991). Laticifers form a complex network of tubes throughout the plant often located just beneath the surface. Wounding by herbivores or other means ruptures the laticifers causing latex, which is stored under pressure, to exude. On exposure to air latex coagulates causing the wound to seal and occluding the mouthparts of insects (Farrell *et al.*, 1991). Indeed, the Monarch butterfly exhibits leaf vein cutting behaviour to reduce the amount of latex it consumes from *Asclepias* (Milkweed) (Dussourd and Eisner, 1987). Latex also has anti-viral (Rafiq *et al.*, 1985) and anti-fungal (Farrell *et al.,* 1991) activities which may be involved in preventing pathogen ingress *via* wounds.

Latex contains a number of components that may be antimicrobial or act to deter herbivores. Traditionally latex from a number of plant species has been used as arrow or fish poisons (Farrell *et al.*, 1991) and a number of secondary metabolites that are toxic to predators or pathogens have been isolated from latex. The opium poppy (*Papaver somniferum*) contains over 20 alkaloids (including morphine and codeine) in its latex (Roberts, 1987), cardiac glycosides are found in milkweed latex (Seiber *et al.*, 1982), diterpenes (Evans and Schmidt, 1976) and non-protein amino acids (Haupt, 1976) are found in *Euphorbia* latex. The latex from lettuce contains a number of sesquiterpene lactones (Sessa *et al.,* 2000). These included lettucenin A, one of the most toxic phytoalexins described, and a number of compounds reported to be insect antifeedants (Sessa *et al.,* 2000). However no correlation was found between the levels of sesquiterpene lactones in latex and resistance to lettuce downy mildew (Sessa *et al.*, 2000).

A number of antimicrobial proteins have been found in latex. Lysozyme and chitinase activities are present in latex from *H. brasiliensis*, papaya, yam, fig, and milkweed (Martin, 1991, Howard and Glazer, 1967, Tsukamoto *et al.,* 1984, Glazer *et al.,* 1969, Lynn, 1989). Chitinases hydrolyse chitin from fungi and insects and have anti-fungal activity *in vitro* (Selitrennikoff, 2001). Many plant chitinases also have lysozyme or lysozyme-like activity against peptidoglycans from bacterial cell walls. Hevamine from *H. brasiliensis* latex had

chitinase and lysozyme-like activity and antibacterial activity *in vitro* (Bokma *et al.*, 1997). Protease inhibitors, which act as insect anti-feedants and anti-fungals, are present in the latex of papaya (El Moussaoui *et al.*, 2001).

Transcripts of the defence related genes chitinase, pathogenicity related-1 (PR-1) protein, phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase and 5-enolpyruvylshikimate 3-phosphate synthase were found to be expressed at 10 to 50 fold higher levels in latex from *H. brasiliensis* than in leaves (Kush *et al.*, 1990). Some of the proteins encoded by these genes have direct antimicrobial activity (chitinase and PR-1) whereas others are involved in the biosynthesis of antimicrobial secondary compounds (PAL and CHS). An EST library of *H. brasiliensis* latex revealed that 12% of the sequences with database matches were defence related (Han *et al.*, 2000). These included protease inhibitors, metallothionein, a WRKY transcription factor and genes induced in response to cold and salt stress in other plants (Han *et al.*, 2000). It appears that latex constitutively expresses a number of genes that are normally stress induced. The role of latex in plants is far from being understood.

1.4.2 Induced defences

When a pathogen breaches the preformed defences of a plant it is either detected and inhibited or killed, or remains undetected long enough to cause disease. Induced defence mechanisms, especially the recognition of pathogens, have been the focus of most of the recent research into plant pathology. The central dogma is that of the interactions between pathogen *avr* (avirulence) genes and corresponding plant *R* (resistance) genes. When a "matching set" of *R* and *avr* genes are present in an interaction the result is a resistance response. If either is inactive or absent disease results (Dangl and Jones, 2001). A large number of *R* and *avr* genes have now been cloned. Avr proteins vary considerably between pathogens but all the *R* genes cloned so far fall into five classes of protein (Dangl and Jones, 2001).

The first *R* gene to be cloned that detects xanthomonads was the rice *Xa21* gene. *Xa21* confers resistance to over 30 races of *X. oryzae* pv. *oryzae* (Song *et al.*, 1995). *Xa21* encodes a transmembrane protein with an extracellular leucine rich repeat (LRR), probably involved in protein-protein interactions, and an intracellular protein kinase domain, probably involved in signalling (Song *et al.*, 1995). Having an extracellular domain, *Xa21* is structurally distinct from other *R* genes that detect bacteria (Hammond-Kosack and Jones, 1997). Because *X. oryzae* pv. *oryzae* is a xylem-invading pathogen it is possible that AvrXa21 (which has yet to be isolated) is an extracellular avirulence protein not delivered by the type III secretion system (Hammond-Kosack and Jones, 1997). Other resistance

oteins active against xanthomonads, including Bs2 (Tai *et al.*, 1999) and Bs4 (Ballvora *et al.*, 2001) from tomato, appear to act within the cell.

The majority of gene-for-gene resistance interactions result in the hypersensitive response (HR). The HR is defined as "The rapid death of plant cells in association with restriction of pathogen growth" (Goodman and Novacky, 1994). The HR is a form of programmed cell death (Lam *et al.,* 2001) and has some similar features to apoptosis but other important differences (Heath, 2000a). The HR leads to brown, dead cells at the infection site; depending on the pathogen and host genotypes this can either affect one or many host cells (Heath, 2000a). Either specific or non-specific elicitors can initiate the HR. The direct or indirect interaction between *avr* and *R* gene products that leads to the HR means that the most likely specific elicitors of the HR are Avr proteins (Heath, 2000a). Non-specific elicitors from fungi include arachadonic acid, cell wall carbohydrates, glycoproteins and proteins (Ebel and Scheel, 1997). The two known non-specific elicitors from bacteria are harpins (Collmer, 1998) and flagellin (Felix *et al.,* 1999).

Ion fluxes, especially the influx of calcium, and the generation of reactive oxygen species (ROS) precede cell death and protein synthesis and an intact actin cytoskeleton appear to be essential for the HR to proceed (Heath, 2000a). In animal cells the mitochondria are essential for triggering apoptosis by sensing calcium levels, pH and the energy status of the cell. Cytochrome C release from the mitochondria leads to the triggering of a protease cascade that leads to cell death (Lam *et al.,* 2001). It is proposed that a similar process may be involved in plants, indeed harpins disrupt mitochondrial function (Xie and Chen, 2000).

Not only does the HR result in an antimicrobial environment in the responding cells, it has roles in the induction of local and systemic resistance (Gilchrist, 1998). The production of reactive oxygen species (ROS) is one of the most immediate downstream effects of the resistance response; indeed ROS are claimed to have a role in triggering cell death (Bolwell, 1999). The generation of ROS is a matter for much debate (Bolwell, 1999) but they are generally agreed to be involved in the direct killing of pathogens, induction of cell wall changes, promotion of the HR and up-regulation of defence gene expression (Lamb and Dixon, 1997).

Induced antimicrobial proteins are often termed pathogenesis related (PR) proteins and are grouped into 14 classes (PR-1 to –14) based on biological activity (Van Loon and Van Strien, 1999). PR-1 proteins accumulate to high levels after pathogen infection and have anti-fungal properties both *in vitro* and in transgenic plants (Niderman *et al.*, 1995). PR-1 proteins have been isolated from a large number of plants and are very similar though their mode of action is unknown (Selitrennikoff, 2001). β1,3-glucanases (PR-2) accumulate rapidly in many plant species in response to pathogen infection, elicitors of defence

response and ethylene (Boller, 1985). β1,3-glucan is a major component of fungal cell walls and it has been shown that β1,3-glucanases can inhibit the growth of fungi *in vitro* (Stintzi *et al.,* 1993) and in transgenic plants (Jach *et al.*, 1995). The antifungal activity of β 1,3-glucanases probably occurs due to weakening the fungal cell wall resulting in cell lysis and death (Selitrennikoff, 2001). PR proteins with chitinases activity are divided into several groups (PR-3, -8 and –11). Several chitinases are potent inhibitors of fungal growth (Schlumbaum *et al.*, 1986). Their antifungal activity is due to the hydrolysis of chitin present in fungal cell walls and, in a manner similar to glucanases, weakening the cell wall and causing cell lysis (Selitrennikoff, 2001). Oligosaccharides released by the action of chitinases can act as elicitors of defence-related genes (Yamaguchi *et al.*, 2000). Chitin binding proteins (PR-4) are thought to have antifungal activity by disrupting fungal cell polarity through an unknown mechanism involving binding to chitin (Bormann *et al.*, 1999). Thaumatin-like proteins (PR-5) have been isolated from a large number of plants and are active against a number of fungi and oomycetes, the precise mechanism of action is unknown though thought to involve increasing membrane permeability (Selitrennikoff, 2001). Proteinase inhibitors (PR-6) have been implicated both as anti-fungals and insect anti-feedants (Ryan, 1990). Induced proteinases (PR-7) have only been detected in tomato and are thought to degrade the fungal cell wall (Van Loon and Van Strien, 1999). Lysozymes (PR-8) degrade the glucosamine and muramic acid components of bacterial cell walls. Most of the known plant lysozymes are bifunctional enzymes also displaying chitinase activity (During, 1993) and provide plants with a general defence against pathogenic bacteria (Rozeboom *et al.,* 1990). Peroxidases (PR-9) are probably involved in strengthening plant cell walls by catalysing lignin deposition (Van Loon and Van Strien, 1999). Ribonucleases (PR-10) are intracellular PR proteins and may be involved in the degradation of viral RNA (Moiseyev *et al.,* 1997). Defensins (PR-12) (Broekaert *et al.*, 1997) and Thionins (PR-13) (Bohlmann, 1994) are small proteins that possess antifungal and antibacterial activity and act at the plasma membrane. Lipid transfer proteins (PR-14) transfer phospholipids between membranes (Garcia-Olmedo *et al.*, 1995). They are active *in vivo* against bacteria and fungi and have been isolated from a number of plants though the precise mechanism of action is not known (Guerbette *et al.*, 1999).

Induced low molecular weight antimicrobials that accumulate in response to infection or stress are termed phytoalexins (Dixon, 2001). The first phytoalexin to be isolated and characterised was pisatin from pea (Cruickshank and Perrin, 1960) and now more than 350 phytoalexins from 30 plant families have been identified (Kuć, 1995). For a phytoalexin to be assigned a role in disease resistance it must be synthesised rapidly in response to infection, accumulate in sufficient quantities to be toxic to the pathogen and be localised

where the pathogen will come into contact with it (Hammerschmidt, 1999). Another indicator of a role for phytoalexins in disease resistance is where phytoalexin detoxifying enzymes are virulence factors for pathogens (VanEtten *et al.*, 2001).

Two antibacterial phytoalexins with a possible role in disease resistance have been isolated. Resistance of soybean to *Pseudomonas syringae* pv. *glycinea* is a gene for gene relationship. Strong correlation between the accumulation of glyceollin and resistance of nine soybean lines to four races of *P. s.* pv. *glycinea* has been demonstrated (Long *et al.*, 1985). Cotton lines resistance to *X. c.* pv. *malvacearum* show accumulation of sequiterpenoid phytoalexins in cells surrounding infection sites at higher levels than those required to inhibit the bacteria *in vitro* (Pierce *et al.*, 1996).

No foliar antimicrobial compounds have been isolated from cassava. However a number of phenolic antimicrobial secondary metabolites have been detected in cassava roots undergoing PPD (Buschmann *et al.*, 2001). Phenolic compounds have also been detected in cassava vascular tissue infected with *Xam* (Kpémoua *et al.*, 1996) and cassava suspension cells treated with fungal elicitors (Gomez-Beeching, 2001). Significantly more phenol-containing phloem and xylem cells were found in cassava plants resistant to CBB than susceptible plants though the antibacterial properties of these compounds were not investigated (Kpémoua *et al.*, 1996). Phenolic compounds are produced in response to a number of stresses and include lignin, suberin and some phytoalexins (Dixon and Paiva, 1995). Phenylalanine ammonia lyase (PAL) is the first enzyme in the phenylpropanoid pathway and is up-regulated in cassava plants infected with incompatible *X. cassavae* but not when infected with compatible *Xam* (Pereira *et al.*, 1999), indicating that phenolic compounds are probably produced in cassava leaves in response to incompatible pathogens.

Plants produce localised cell wall changes in response to pathogens. These include papillae that contain a mixture of components including callose, phenolics and hydroxyproline-rich glycoproteins (Brown *et al.*, 1995). Papillae have been observed in cassava vascular tissue infected with *Xam* (Kpémoua *et al.*, 1996). Papillae formation appears to be inhibited by *X. c.* pv. *vesicatoria* and *Pseudomonas syringae* pv. *phaseolicola* in a *hrp* gene dependent manner (Brown *et al.*, 1995, Bestwick *et al.*, 1995). The role of papillae is probably to prevent pathogen spread.

Lignification of the cell wall occurs in many plants as a specific response to infection by pathogenic fungi (Ride, 1983). Lignin may have several roles in preventing pathogen spread. It may provide a physical barrier against the pathogen, make the cell wall impervious to pathogen wall-degrading enzymes, block transfer of water or nutrients to the pathogen and block transfer of toxins from the pathogen to the plant cell. In a more direct

way it may produce intermediates toxic to the pathogen or inhibit pathogen growth by lignifying pathogen cell walls (Ride, 1983). Lignification and suberisation of vascular tissues in *Xam* infected cassava plants has been detected (Kpémoua *et al.,* 1996). Two enzymes involved in the production of lignin, PAL and peroxidase, are up-regulated in cassava leaves infected with incompatible *X. cassavae* (Pereira *et al.*, 1999, Pereira *et al.,* 2000). These enzymes are not induced to such a great extent in response to *Xam* implying that *Xam* may be suppressing the cassava defence response (Pereira *et al.*, 2000).

Xam is a vascular pathogen and the production of vascular occlusions has been reported in infected cassava plants (Kpémoua *et al.,* 1996). The two main forms of vascular occlusions are tyloses and gels. Tyloses are balloon-like enlargements of vascular parenchyma cells that project through pits in the vessel walls to block the xylem (Crute *et al.*, 1985). Tyloses have been reported in cassava infected with *Xam* although no significant difference was found between the numbers in susceptible and resistant plants (Kpémoua *et al.,* 1996). Infected cassava xylem vessels were plugged with pectin- or lignin-based gels that coincided with restricted spread of the pathogen (Kpémoua *et al.*, 1996). These gels also showed accumulations of phenolic compounds, however no significant difference was found between resistant and susceptible varieties and no proof was presented that the plug prevented pathogen spread (Kpémoua *et al.,* 1996).

1.5 Aims

The aims of this thesis are to investigate pathogenicity determinants of *Xam* and defence mechanisms of cassava. Three areas are investigated in the following chapters; pathogenicity determinants of *Xam*, preformed defence mechanisms of cassava and induced defence mechanisms of cassava. The pathogenicity of the *Xam* strain used in this study and the resistance of three cassava cultivars was also investigated.

Two pathogenicity factors are investigated, extracellular polysaccharide (EPS) and pectate lyase (Pel). EPS is a virulence factor in a number of phytopathogenic bacteria (Denny, 1995) and EPS from *Xam* has been localised in infected cassava plants (Boher *et al.,* 1997). Degradation of pectin has been reported in infected cassava plant (Boher *et al.*, 1995) and Pel was the only significant pectolytic enzyme produced by *Xam* grown on cassava cell walls (Deshappriya, 1992). The aim is to produce a defined mutant of *Xam* that produces no EPS and investigate the effects on pathogenicity. The production of Pel by *Xam* will also be investigated with the aim of producing a defined mutant.

The preformed components of cassava resistance to bacterial blight will be investigated. Three potential preformed defences will be looked at; stomatal density and distribution, antimicrobial properties of cassava latex and preformed antibacterial compounds present in

cassava leaves. *Xam* is assumed to enter cassava leaves through the stomates before spreading to give systemic infection (Maraite, 1993). The density and distribution of stomates on a number of cassava cultivars will be investigated to see if it correlates with resistance to CBB. On wounding cassava exudes copious quantities of latex. Latex from a number of different species contains antimicrobial proteins (e.g. El Moussaoui *et al.*, 2001, Martin, 1991), antimicrobial compounds (e.g. Sessa *et al.,* 2000) and is assumed to play a role in defence against herbivores and pathogens (Farrell *et al.*, 1991). Potential antimicrobial enzymes in cassava latex will be assayed and an EST library of cassava latex will be made to identify genes that may be involved in defence. With the exception of cyanogenic glycosides no foliar antimicrobials have been isolated from cassava leaves. Low molecular weight compounds with activity against *Xam* will be isolated from cassava leaves and characterised.

Very few cassava genes induced or repressed in response to biotic challenge have been isolated (Verdier *et al.*, 1997). In order to identify genes involved in cassava response to biotic challenge a method of differential display have been utilised. cDNA-AFLP has been used in a number of systems to discover genes involved in plant development and response to stress (Durrant *et al.*, 2000, Cooper, 2001, Campalans *et al.*, 2001), including genes involved in post-harvest deterioration of cassava roots (Huang *et al.*, 2001). By utilising non-host phytopathogenic bacteria to induce the HR on cassava some of the difficulties associated with investigating the slow and incomplete expression of resistance to *Xam* and isolate genes important in cassava disease resistance will be avoided.

This thesis aims to further the understanding of cassava bacterial blight. By looking at both the defence mechanisms of the plant and the pathogenicity determinants of the pathogen it is hoped that some leads may develop to help improve resistance to this disease. Improved resistance to CBB would minimise the effect this disease can have on a population dependent on cassava as a major source of calories.

Chapter 2

Materials and Methods

Described here are general materials and methods, specific to more than one results chapter. Where methods are only applicable to one chapter separate materials and methods are provided. Unless stated chemicals were of AnalaR grade obtained from Merck Eurolabs Ltd., Poole, Dorset, UK.

2.1 Plant material, growth and maintenance

In total six cassava cultivars were grown for this study (table 2.1). MCol 22, MNgal, MNga 19 and MVen 77 were cultivated from stocks at the University of Bath. Cultivars CM 2177-2 and MNga 2 were propagated from stakes sent by CIAT, Colombia. Stem stakes were cut into 10-15cm lengths and their lower ends dipped into a commercial rooting powder containing IBA (Seradex, May and Baker Ltd.). Stem cuttings were planted in a mixture of compost and perlite (3:1 v/v) in 12.5cm diameter plastic pots. Stems were maintained with high humidity (80%) at 28-30°C until shoots and roots were initiated. Stems were then transferred to 20cm diameter pots containing C2 compost and a slow release fertiliser (Vitafeed 101). Plants were grown under glass at 25-30°C with natural lighting supplemented by mercury lamps when required. Humidity was kept high, both to encourage pathogen development and deter red spider mite.

2.2 Phytopathogenic Bacteria

Eight isolates of *Xanthomonas axonopodis* pv. *manihotis* (*Xam)* were used in this study (table 2.2). All strains isolated after 1990 were provided by Dr. K. Wydra, University of Gottingen, Germany. All strains isolated prior to 1990 were from the University of Bath stock collection. *Pseudomonas syringae* pv. *tomato (Pst)* strain DC3000 was provided by Prof. John Mansfield (Imperial College at Wye, UK) and *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) was from the University of Bath stocks.

2.2.1 Growth of bacteria

NYGA (see appendix 1) plates streaked with *Xam* were incubated at 28°C for 48 h. Individual colonies were transferred to NYGB (see appendix 1) grown at 28°C with shaking (200rpm) for 24 h. *Pst* and *Xcv* were treated as above but grown at 25°C.

2.2.2 Maintenance and storage of bacterial cultures

Bacterial strains were maintained on NYGA at room temperature for short periods of time (2-3 weeks) (storage of *Xam* at 4°C resulted in a rapid loss of viability). Long-term

Cultivar	Response to CBB*	Details	Notes
CM 2177-2	Intermediate	Controlled cross	Male parent of genetic map
MCol22	Susceptible	Colombian land race	
MNga1		Nigerian land race	
MNga2	Tolerant	Nigerian elite variety	Female parent of genetic map, resistance integrated from M. glaziovii
MNga19	Tolerant	Nigerian elite variety	Resistance integrated from M. glaziovii
MVen77		Venezuelan land race	

Table 2.1 Cassava cultivars grown for this study
* The resistant reaction to CBB is extremely variable (see introduction and chapter 3 for more details). Where given a response has been determined by combining data from Sanchez *et al.*, 1999 and Restrepo *et al.*, 2000.

Isolate	Year of isolation	Country of isolation
I56	1993	Nigeria
246	1993	Nigeria
1222	1984	Colombia
2368	1994	Uganda
2507	1993	Nigeria
2762	1993	Nigeria
2967	1972	Brazil
3039	1976	Colombia

Table 2.2 *Xanthomonas axonopodis* pv. *manihotis* strains used in this study.

storage was as frozen glycerol stocks. Overnight cultures were grown in NYGB (see appendix 1) then 20% v/v sterile glycerol was added and the stock stored at –70°C. When required a sterile inoculation loop was scraped across the frozen stock and streaked onto fresh NYGA plates.

2.2.3 Growth curve of *Xam*

Fifty ml NYGB were inoculated with *Xam* and grown overnight. The bacteria were harvested by centrifugation (3000g for 15 min) and washed twice in sterile distilled water pH 7.0. The resulting pellet was resuspended in sterile distilled water and the optical density at 600nm determined using a Shimadzu UV-260 spectrophotometer. The bacterial suspension was then diluted to give a range of absorbance values at 600nm between 0 and 1 OD units. The resulting diluted suspensions were then serially diluted to give dilutions at 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}. One hundred microlitres of each dilution were spread onto NYGA plates and incubated for 48 h at 28°C. Numbers of resulting colonies were counted in order to determine the relationship between Optical Density and Colony Forming Units per ml (cfu ml^{-1}) (figure 2.1).

2.3 Inoculations of plants

Bacteria were grown overnight in NYGB and harvested by centrifugation (3000g for 15 min). Bacteria were washed once in either sterile distilled water (pH 7) or 10mM $MgSO_4 \cdot 7H_2O$ (pH 7) and adjusted turbidometrically to give the required concentration.

2.3.1 Syringe inoculation

A 1ml disposable syringe without a needle was used to infiltrate bacteria into the abaxial surface of leaves. Pressure was applied to the syringe until visible water soaking could be seen. Symptom progression was monitored and scored on a 0-5 scale where 0 = No symptoms, 1= water-soaking, 2=chlorosis, 3=spreading chlorosis, 4= wilt of infected lobes and 5= wilt of whole leaf (figure 2.2).

2.3.2 Petiole stab

A syringe with a needle was stabbed in the petiole at the base of a leaf. Pressure was applied to force bacteria into the wound. Resulting symptoms were recorded as a 0-5 scale where 0 = no symptoms, 1 = ooze exuding from wound, 2 = ooze spreading from initial site of infection, 3 = necrosis round infection site, 4 = wilt of individual lobes and 5 = wilt of whole leaf.

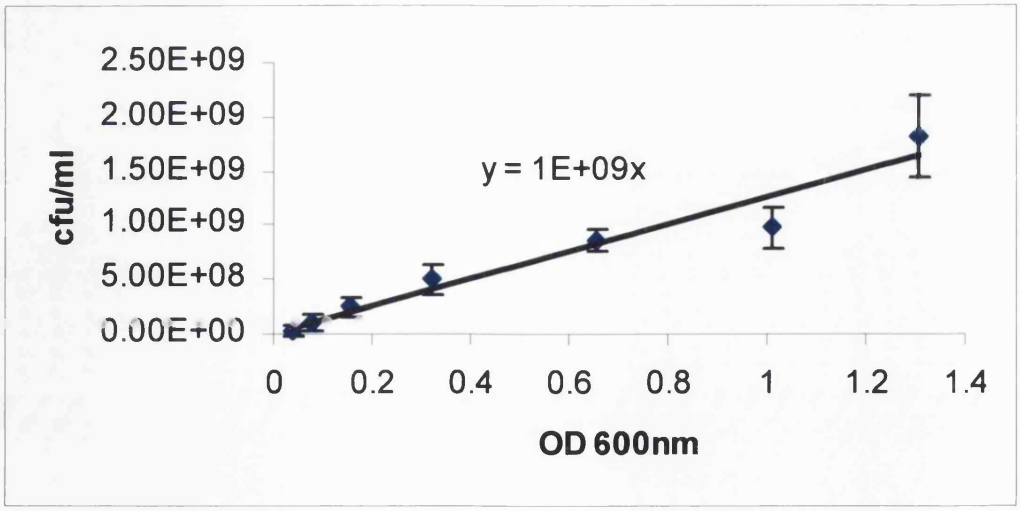
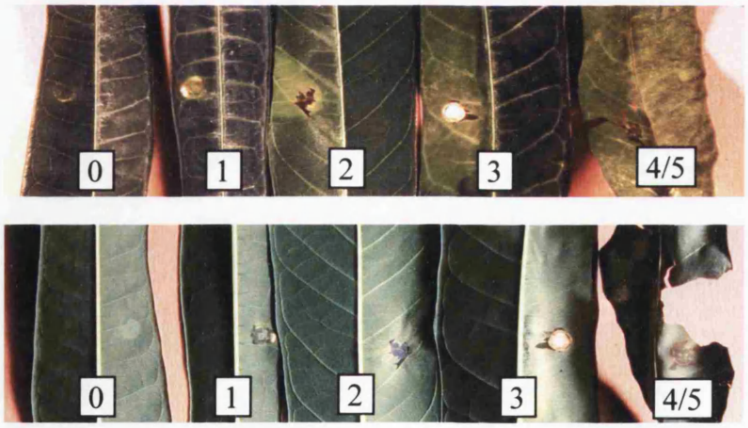


Figure 2.1 Relationship between optical density at 600nm and colony forming units (CFU) per ml of *Xam*

An overnight *Xam* culture was diluted to specific optical densities at 600nm in NYGB medium. The cultures were then serially diluted, plated to NYGA plates and incubated for two days at 30°C. The number of resulting colonies was then determined. The graph above shows the relationship between OD600nm and cfu/ml. Each point is the mean of six replicates, error bars show standard deviation from the mean. The formula on the graph indicated that an OD of 1 = ca. $1x10^9$ cfu/ml.



Upper surface

Lower surface

Figure 2.2 Scale of Symptom Index (SI) caused by *Xam* infiltration.

After infiltration with *Xam* leaves were scored on a 0-5 scale depending on the symptoms observed. 0= no symptoms 1= water soaking (this is clearer on the lower surface of the leaf) 2= chlorosis (though in the leaf above the chlorosis has started to spread) 3= spreading chlorosis 4= wilt of a lobe and 5= wilt of the whole leaf.

2.3.3 Bacterial numbers in infected leaves

A disc of leaf material was removed using the lid of a sterile microfuge tube. The lid was placed over the infected area and pushed against the leaf until it cut through and removed a leaf disc. The tube was then shut sealing the leaf disc inside. 1 ml of sterile distilled water (SDW) pH 7 was added to the tube and vortexed to remove the majority of surface contaminants. Occasionally the leaf disc stuck to the top of the tube, in these cases gentle centrifugation (3000g for 1 min) was used to dislodge the disc. The SDW was removed with a suction line. The leaf disc was then ground in 100µl 10mM $MgSO_4 \cdot 7H_2O$ (pH 7) using a sterile mini pestle (Sigma-Aldrich Ltd., Poole, Dorset, UK). This suspension was then serially diluted in a sterile microtitre plate. NYGA or CTA (see appendix 1) plates were divided into four and three spots of 20µl from a dilution were plated onto a quarter-plate (a total of 12 spots from four different dilutions per plate). Plates were incubated at 28°C for 48 h before counting.

2.4 Molecular Biology

The majority of techniques used were adaptations from Sambrook *et al.,* (1989) or Ausubel *et al.*, (1990). Again specific techniques are detailed in individual chapters.

2.4.1 Transformation of *E. coli*

For the majority of this study *E. coli* strain DH5α. (Stratagene Ltd., Amsterdam, The Netherlands) was used. *E. coli* cells were grown at 37°C in LB medium (see appendix 1). Cells were made transformation competent essentially as detailed in Hanahhan (1983). A single colony was grown in 250ml SOB broth (see appendix 1) to an OD of 0.4. Cells were pelleted by centrifugation at 3000g for 10 min at 4°C and resuspended in 100ml ice cold FSB buffer (10mM potassium acetate, 100mM KCl, 10mM $CaCL_2 \cdot 2H_2O$, 45mM $MnCl \cdot 4H_2O$, 3mM hexaminocolbalt chloride, 15% (v/v) glycerol, pH 6.4). The cells were incubated on ice for 10 min, re-centrifuged at 3000g for 10 min and resuspended in 15ml ice cold FSB. 500µl of DMSO was added and the cells incubated on ice for 15 min. Aliquots were snap frozen on dry ice and stored at –70°C.

Transformation efficiency of cells was tested by the addition of 0.5ng of pUC18 DNA to 100µl competent cells previously thawed on ice. Cells were incubated with the DNA on ice for 30 min before heat shocking at 42°C for 90s followed by a 2 min incubation on ice. 800µl of LB medium was added and cells were incubated at 37°C shaking at 200 rpm for 1h to allow expression of antibiotic resistance genes. Aliquots of the transformed cells were then lawned onto LB medium supplemented with the appropriate antibiotics and

incubated at 37°C overnight. Competent cells prepared using this method routinely had an efficiency of over $1x10^7$ transformants per μg plasmid DNA.

2.4.2 Plasmid minipreps

Single colonies were inoculated into 5 ml LB broth supplemented with the appropriate antibiotics and grown overnight at 37°C with shaking at 200 rpm. Cells from 1.5 ml of overnight culture were harvested by centrifuging in a microfuge at maximum speed for 30s. The supernatant was removed with the aid of a suction line and cells resuspended in 300μl resuspension buffer (50mM Tris, 10mM EDTA pH 8). 300μl of lysis solution was added (0.2M NaOH, 1% w/v SDS) and mixed thoroughly by inverting the tube several times before incubating at room temperature for 5 min. Genomic DNA and proteins were then precipitated with the addition of 300μl ice cold precipitation buffer (3M potassium acetate, 28.5% v/v glacial acetic acid, pH ≈ 4.4) and incubating on ice for 5 min. Tubes were centrifuged at maximum speed in a microfuge for 10 min to pellet genomic DNA and proteins. 750μl of supernatant was removed to a fresh tube and an equal volume of Tris saturated phenol (pH 8.0): chloroform: isoamyl alcohol (25:24:1) was added. Tubes were mixed thoroughly by vortexing and centrifuging at maximum speed for 5 min separated the two phases. The aqueous layer was removed to a fresh tube and DNA precipitated with the addition of 0.5ml propan-2-ol. DNA was collected by centrifugation at maximum speed for 10 min, washed once in 70% ethanol and air-dried. DNA was dissolved in 50μl sterile MilliQ water. This DNA was of a quality comparable to that obtained using plasmid preparation kits and was used for the restriction digests, PCR and sequencing.

2.4.3 Agarose gel electrophoresis

TBE gels (Tris Borate EDTA)

Agarose (between 0.8 and 2% (w/v) depending on the size of DNA under analysis) was melted in 1xTBE (90mM Tris, 90mM boric acid, 1mM EDTA) in a microwave and cooled to *ca.* 55°C. Ethidium bromide was added to a final concentration of 0.2 μg ml^{-1} prior to pouring the gel. Gels were run on an Anachem submerged gel electrophoresis tank as manufacturers instructions (Anachem, Luton, UK) Samples were mixed with 5x blue loading buffer (0.25 (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 50% (v/v) glycerol) prior to loading. Where the size of DNA was expected to co-migrate with the dyes (for a 1% agarose gel *ca.* 500bp xylene cyanol and *ca.* 3kb bromophenol blue (Sambrook *et al.*, 1989)) 5x orange loading buffer (0.25% (w/v) orange G, 50% (v/v) glycerol) was used as that dye migrates rapidly through the gel. Depending on the resolution required gels were either run at 30v (high resolution, e.g. for southern blots) or

100v (low resolution, e.g. for analysing PCR products) in 1xTBE until the loading dyes had migrated the appropriate distance into the gel. DNA molecular weight markers (normally 0.5µg of 1kb ladder (New England Biolabs, Hitchin, Hertfordshire, UK)) were run alongside samples. Gels were viewed under an UV transilluminator (UVP, Cambridge, UK) and images captured using a CCD camera (UVP) and Grabit software (Synoptics, Cambridge, UK). Images were printed using a digital graphic printer (Sony, Weybridge, Surrey, UK). In addition to analysing DNA, TBE gels were used to assess to integrity of RNA samples from cassava tissue.

2.4.4 Polymerase Chain Reaction (PCR)

PCR was performed routinely to amplify specific DNA sequences. Whilst a number of different sources of Taq polymerase were used throughout the work most success was found using Ready red Taq mix (Sigma-Aldrich). Unless stated all PCR reactions were carried out in a PTC-100 programmable thermal cycler (MJ research, Watertown, MA, USA). With the exception of commercial primers and those used for AFLP all primers were designed by hand using the following rules;

1) Final length of each primer was between 20-30 oligonucleotides
2) G+C content was between 40-60%
3) Pairs of primers were of similar length and similar G+C content
4) Runs of bases were avoided
5) Either a G or a C was used if possible for the final base

All primers were obtained from Invitrogen (Paisley, UK) and dissolved in sterile MilliQ water to a final concentration of 50mM. The sequences of all primers used in this study are in appendix 3.

Most reactions were carried out in a final volume of 50µl with 50pmol of each primer and 1x ready mix. Template concentrations varied with the template used. For colony PCR single *E. coli* colonies were picked with a sterile pippettor tip and transferred to 50µl PCR reaction.

Cycling conditions were generally

3 min denaturation at 95°C

30 s denaturation at 95°C
30 s annealing at 55°C
1 min per kilobase of target fragment extension at 72°C
} 35 cycles

5 min final extension at 72°C

10µl of reaction was analysed by gel electrophoresis. Where non-specific amplification was observed annealing temperature was increased and where no amplification product was observed annealing temperature was decreased.

2.4.5 Construction of cDNA and genomic libraries

2.4.5.1 cDNA synthesis

cDNA libraries were constructed using the ZAP-cDNA synthesis kit (Stratagene) essentially as manufacturer's instructions. Unless stated, all components were provided with the kit. Polyadenylated RNA was isolated from total RNA using the PolyATract system (Promega, Southampton, UK). Secondary structure was removed from RNA by heating to 95°C for 2 min and cooling rapidly on ice. First strand cDNA synthesis was carried out using reverse transcriptase, primed with oligo dT with a *Xho* I site. The nucleotide mixture included methylated dCTP to allow cleavage of the *Xho* I site without digesting the cDNA. RNaseH treatment removed the mRNA and second strand synthesis was carried out using DNA polymerase I primed with random hexamers. 2µl 800Ci/mmol [α–^{32}P] dATP (ICN Pharmaceuticals, Basingstoke, UK) was added to the second strand synthesis reaction to allow size fractionation after cDNA synthesis was complete. *Eco* RI adapters were ligated to the ends of the cDNA and phosphorylated with T4 polynucleotide kinase. The *Xho* I restriction site in the oligo dT was cleaved with *Xho* I and the cDNA was size fractionated through a drip column containing Sepharose CL-2B (Amersham Biosciences, Little Chalford, Buckinghamshire, UK). Fractions were analysed on a 1xTBE 5% nondenaturing polyacrylamide gel (Sambrook *et al.*, 1989) using a mini-protan II gel apparatus (Bio-Rad, Hemel-Hempsted, Herts, UK). Gels were dried with a gel drier (Bio-Rad) and exposed to X-Omat AR film (Eastman Kodak, Rochester, NY, USA). Fractions with the highest molecular weights were pooled and ligated into the Uni-ZAP XR vector.

2.4.5.2 Ligating into Uni-ZAP XR and packaging into phage

Digested DNA fragments (either cDNA or total genomic digest) were mixed in an equimolar ratio with pre-digested Uni-ZAP XR vector (Stratagene). Ligations were performed at 4°C for two days in a total volume of 5µl with 2U T4 DNA ligase (Stratagene) in 1 x ligase buffer supplemented with 1mM rATP. 1µl of the ligation was packaged into phage using the Gigapack III gold packaging extract (Stratagene) as manufacturer's instructions. The packaging reaction was stopped with the addition of 500µl SM buffer (100mM NaCl, 10mM $MgSO_4$, 50mM Tris-HCl (pH 7.5) and 0.01% gelatine) and 20µl chloroform.

2.4.5.3 Titrating the library

The titre of the library was determined by incubating 1µl and 0.1µl of the packaged phage with 200µl of an OD_{600} 0.5 suspension of XL-1 Blue *E. coli* (Stratagene) cells in 10mM $MgSO_4 \cdot 7H_2O$. The phage and bacteria were incubated at 37°C for 15 min to allow the phage to attach to the cells. 3ml of NZY top agar at 48°C (see appendix 1), 15µl of 0.5M IPTG (in H_2O) (Melford Laboratories, Ipswich, UK) and 50µl 250mg ml^{-1} X-gal (in DMF) (Melford Laboratories) were added to the phage and bacteria prior to plating to 9cm NZY agar plates. After the top agar had set plates were incubated at 37°C overnight. Background plaques containing no insert were blue whereas plaques containing inserts were clear. The number of plaques was counted and the titre of the primary library determined.

2.4.5.4 Amplification of libraries

Primary libraries were amplified to increase the stability of the libraries. *ca.* $5x10^4$ pfu of bacteriophage with 600µl of an OD_{600} 0.5 suspension of XL-1 Blue MRF' *E. coli* cells for 15 min at 37°C. 6.5ml of NZY top agar at 48°C was added and the suspension plated to 150mm NZY plates. Plates were incubated at 37°C for *ca.* 8 h so that plaques were touching but no bigger than 2mm. Plates were then overlaid with 10ml SM buffer and incubated overnight at 4°C with gentle rocking to allow the phage to diffuse into the buffer. The phage suspension was removed to a polypropylene tube and *E. coli* cells lysed with the addition of 5% (v/v) chloroform and a 15 min incubation at room temperature. *E. coli* cell debris was removed by centrifugation at 500g for 10 min. The extraction was repeated on the supernatant and chloroform added to a final concentration of 0.3% (v/v) before storing the amplified library at 4°C. Aliquots of the library were also stored at –70°C in 7% (v/v) DMSO. The titre of the amplified library was checked as above.

2.4.5.5 Screening libraries

The libraries were plated out at $5x10^4$ pfu $plate^{-1}$ onto 150mm NZY agar plates as detailed above. After incubating at 37°C for 8 h the plates were stored at 4°C overnight to prevent the membrane sticking to the top agar. Hybond N+ membranes (Amersham Biosciences) were used to lift plaques as manufacturer's instructions; membranes were oriented on the plate by a series of pinpricks. DNA was fixed to the membranes using a UV transilluminator (Sambrook *et al.,* 1989).

DNA probes were labelled with [α-^{32}P]dCTP (ICN) using an oligolabelling kit (Amersham Biosciences) as manufacturer's instructions. Unincorporated radionucleotides were removed using a Sephadex G-50 (Amersham Biosciences) spin column (Sambrook *et al.*

1989). Hybridization was performed using the protocol of Church and Gilbert (1984). Membranes were washed in 0.1% SDS and 0.1xSSC twice for 15 min at 65°C before exposure to X-OMAT film (Eastman Kodak). Plaques of interest were picked using a sterile pipette tip and transferred to 500µl SM buffer with 20µl chloroform and stored at 4°C.

2.4.5.6 Excision of phagemids

The Uni-ZAP XR vector allows excision of the pBluescript phagemid with the ExAssist helper phage. 200µl of XL1-Blue MRF' *E. coli* cells at an OD_{600} of 1 in 10mM $MgSO_4 \cdot 7H_20$ were incubated with *ca.* $1x10^5$ phage particles (either from a single plaque or from the entire library) and $1x10^6$ ExAssist helper phage at 37°C for 15 min. 3 ml LB broth was added and the excision incubated at 37°C for 3 h with shaking at 200rpm. The excision was then heated to 70°C for 20 min and *E. coli* cell debris removed by centrifugation at 1000g for 15 min. The filamentous phage particles were then stored at 4°C. 200µl of an OD_{600} 1 suspension of SOLR *E. coli* cells in 10mM $MgSO_4 \cdot 7H_2O$ were infected with 100µl of filamentous phage particles and incubated at 37°C for 15 min. 200µl of the cell mixture was then plated onto LB agar containing 50µg ml^{-1} ampicillin (Melford Laboratories) and incubated overnight at 37°C.

2.5 Bioinformatics

Sequence information resulting from this study was analysed by a variety of methods. To identify homologies, sequences were compared to the Genbank database using BlastX or BlastN (Altschul *et al.*, 1997). Sequences were assembled using the GCG Wisconsin package available at the University of Bath. A large number of manipulations (including sequence alignments, identifying protein features, translations and restriction enzyme mapping) were performed on the San Diego Supercomputing Centre Biology Workbench web site. The Human Genome Mapping Project web site also provided some useful programmes, especially PIX where a large number of programmes are run on a sequence.

Chapter 3

Resistance of Cassava to Bacterial Blight

3.1 Introduction

The only practicable method of controlling cassava bacterial blight is by the introduction of resistant cassava cultivars (Lozano, 1989). Three cultivars were tested for resistance in this study; CM 2177-2, MCol22 and MNga2. MCol22 was selected because it has been investigated extensively in the past and is generally agreed to be susceptible to CBB (Pereira *et al.,* 1999). CM 2177-2 and MNga2 (also known as TMS 30572) were selected because they are the male and female parents respectively of a cross used to construct a genetic map of cassava (Fregene *et al.*, 1997). A number of groups collaborated as part of the EU grant that funded this work and it was pertinent to work with the same cultivars. Any genes of interest to CBB resistance can be added to the map and areas of the genome that are involved in resistance to CBB characterised.

The resistance levels of CM 2177-2 and MNga2 to CBB appear very similar (but see below) though they have high levels of DNA polymorphism (Gomez *et al.*, 1996) indicating that cassava has many mechanisms of resistance to CBB. The high level of polymorphism between the genomes of the two cultivars was the main reason for selecting them as the parents of the map (Fregene *et al.,* 1997). The cultivars are polymorphic for a number of other traits; MNga2 is resistant to African cassava mosaic virus (ACMV), CM 2177-2 is resistant to the cassava mealy bug and has good cooking qualities and both are adjusted to different edaphoclimatic zones (ECZ) (Fregene *et al.*, 1997). MNga2 also contains genes introgressed from the wild relative of cassava *M. glaziovii* which has been used to improve the cassava germplasm for various traits, including resistance to CBB (Hahn *et al.*, 1979). Finally, the two cultivars were selected for political reasons, with one cultivar originating from South America (CM 2177-2) and one from Africa (MNga2).

Unfortunately widely different reaction to CBB was not considered during selection of parents for the cross and both MNga2 and CM 2177-2 are described as tolerant to CBB in the original paper describing the genetic map (Fregene *et al.*, 1997). Progeny do not show good segregation for reaction to *Xam*, although attempts have been made to map resistance to CBB using the F1 population (Jorge *et al.,* 2000; Jorge *et al.*, 2001). CBB resistance is notoriously difficult to quantify and there has been conflict as to the disease reactions of the parents. Table 3.1 is a summary of data or claims of resistance from several recent papers on CBB. Of ten separate experiments, half found MNga2 to be more resistant to CBB than CM 2177-2 and half found CM 2177-2 to be more resistant than MNga2 (Table 3.1). Where tested, MCol22 appeared to be more susceptible than either CM 2177-2 or MNga2 but not necessarily than both (table 3.1). Resistance appears to be dependent on

pathogen strain, inoculation method and, in field trials, the ECZ (Table 3.1). It is evident that differences between an interaction classed as resistant to CBB and an interaction classed as susceptible is often slight. For example Restrepo and co-workers (2000) scored plants on a 0-5 scale based on visible symptoms. Cultivars with a mean score of ≤ 3 are grouped as resistant and those with a mean score >3 are grouped as susceptible (Restrepo *et al.*, 2000). MNga2 plants grown in ECZ 1 in 1998 had a mean disease reaction of 2.9 and were classed as resistant whereas MCol22 plants in the same experiment were classed as susceptible even though they had a similar mean disease reaction of 3.3 (Restrepo *et al.*, 2000). In the papers detailed in table 3.1 all cassava cultivars show some symptoms when infected with *Xam,* with resistance being apparent as either a delay in the onset of symptoms or arrested symptom development.

In the light of conflicting reports it was decided to test the resistance of the three cultivars to *Xam* strain I56. Various methods for inoculating cassava with *Xam* exist including the spraying of inoculum onto leaves, abrasion of leaf surfaces followed by application of inoculum, stem puncture, petiole stabbing, leaf clipping, leaf infiltration and applying inoculum into wounds on the leaf surface (Terry, 1976; Flood *et al.,* 1995; Restrepo *et al.*, 2000a). The method of inoculation is often critical in determining resistance. Introduction of 26 strains of *Xam* into CM 2177-2 and MNga2 by stem stabbing resulted in the classification of CM 2177-2 as susceptible to all of the strains and MNga2 as resistant to ten strains (Restrepo *et al.*, 2000a). However when the same 26 strains were introduced *via* leaf wounds CM 2177-2 was significantly more resistant than MNga2 to all strains tested (Restrepo *et al.*, 2000a).

In addition to testing for resistance of cassava to *Xam* it is pertinent to test that the strain of *Xam* to be used in the work is pathogenic. Strain I56 was isolated from Nigeria in 1993. Whilst the parents of the genetic map are rapidly becoming the cassava cultivars to investigate no strain of *Xam* has become universally utilised. I56 was chosen for this study because it was isolated from West Africa, where CBB has the potential to cause real devastation, is highly virulent in the field (K. Wydra, personal communication) and is the most amenable strain to tetrazolium based bioassays (see chapter 5, this thesis). African isolates of *Xam* are generally less diverse than South American isolates (Verdier *et al.*, 1993) and it is more likely that one African strain will be representative of the population of bacteria in the wild than a single South American strain.

The two inoculation methods that have been most reproducible at the University of Bath are leaf infiltration and petiole stabbing (R. Cooper personal communication). These methods were used to test the resistance of CM 2177-2, MCol22 and MNga2 to *Xam* strain I56. Leaf infiltration without wounding mimics natural infection through stomates whereas

Method [a]	Strains [b]	Conditions [c]	CM 2177-2 [d]	MCol22 [d]	MNga2/TMS [de]	Most resistant	Reference
Literature			Tolerant	nd	Tolerant	-	Fregene et al., 1997
Stem stab	10	Greenhouse	S. 6 strains R. 4 strains	S. 10 strains	S. 3 strains R. 7 strains	MNga2	Verdier et al., 1998
Literature			nd	Susceptible	Resistant	MNga2	Pereira et al., 1999
Stem stab	2	Greenhouse	R. 1 strain S. 1 strain	R. 1 strain S. 1 strain	S. 2 strains	CM 2177-2 / MCol22	Sanchez et al., 1999
Stem stab	5	Greenhouse	+ MN. 1 strain - MN. 3 strains = MN. 1 strain	nd	+ CM 3 strain - CM 1 strain = CM 1 strain	MNga2	Jorge et al., 2000
Stem stab	1	Greenhouse	Susceptible	Susceptible	Resistant	MNga2	Restrepo et al., 2000
Field	Natural	ECZ 2 1998	Resistant	Susceptible	Susceptible	CM 2177-2	Restrepo et al., 2000
Field	Natural	ECZ 1 1997	Resistant	Susceptible	Resistant	MNga2	Restrepo et al., 2000
Field	Natural	ECZ 2 1997	Susceptible	Susceptible	Susceptible	CM 2177-2	Restrepo et al., 2000
Stem stab	26	Greenhouse	S. 26 strains	S. 25 strains R. 1 strain	S. 16 strains R. 10 strains	MNga2	Restrepo et al., 2000a
Leaf wound	26	Greenhouse	+ MC. all strains + MN. all strains	- CM. all strains = MN. most strains	- CM all strains = MN most strains	CM 2177-2	Restrepo et al., 2000a
Field	Natural	ECZ 2	+ MN.	Nd	- CM	CM 2177-2	Jorge et al., 2001

[a] Method used in the paper to infect plants, "Literature" = no experimentation in the paper but claims to resistance are made. [b] The number of strains used in artificial inoculations. [c] In field trials the ECZ = Edaphoclimatic zone where experiments took place. [d] + = more resistant than, - = less resistant than and = = equal reistance to. S = susceptible and R= resistant as stated in the original papers, MN= MNga2, CM= CM 2177-2, MC = MCol22, nd = not determined. [e] TMS 30572 is the same as MNga2,

Table 3.1 Resistance of three cultivars of cassava to *Xam* as determined or claimed in several recent papers.

injection into petioles mimics wounding and direct introduction to the xylem. Inoculum concentration can be critical in revealing level of isolate pathogenicity or of host resistance. Therefore for leaf infiltration two levels of inoculum were used; the lowest had been shown to be the lower limit of that required to cause symptoms (Flood *et al.*, 1995).

3.2 Materials and Methods

3.2.1 Cassava Cultivars and Inoculation Methods

CM 2177-2, MNga2 and MCol22 were propagated from stakes as described in chapter 2. Plants were inoculated with *Xam* strain I56 resuspended in 10mM $MgSO_4 \cdot 7H_2O$ as described in chapter 2.

3.2.2 Experimental design

Fifteen plants of each cultivar were used in this study. They were randomly distributed in the glasshouse and treated identically. Day temperature was *ca.* 27°C and night temperature *ca.* 15°C. Humidity was kept high by frequent watering and filling large troughs placed round the glasshouse with water. Each plant had two petioles stabbed with a syringe and needle and injected with *ca.* 0.1 ml $1x10^6$ cfu ml^{-1} *Xam*, two leaves infiltrated with $1x10^6$ cfu ml^{-1} *Xam* and two leaves infiltrated with $1x10^4$ cfu ml^{-1} *Xam.* Plants also had control treatments of one petiole stabbed with 10mM $MgSO_4 \cdot 7H_2O$ and one leaf infiltrated with 10mM $MgSO_4 \cdot 7H_2O$. Leaves were infiltrated at single points on three lobes.

Disease progression was monitored frequently and symptoms recorded as detailed in chapter 2.

3.3 Results

Leaves infiltrated with $1x10^6$ cfu ml^{-1} began to show symptoms 6d post inoculation (dpi) (figure 3.1). Symptoms were initially persistent water-soaking observed at the site of infection. The appearance of a chlorotic halo around the infection point, spreading necrosis and eventual wilting of leaves (figure 2.2) followed water-soaking symptoms. Systemic symptoms were not observed in any of the infected plants with leaves abscising within 3-7 d of wilting. Symptoms first appeared on MCol22 and by 8dpi the majority of MCol22 plants showed water soaking (Symptom Index (SI) of 0.82±0.31). By 11dpi the majority of plants from all cultivars showed water soaking (figure 3.1). MCol22 plants showed significantly more symptoms than both MNga2 and CM2177-2 at 8, 15 and 27dpi when data sets were compared with a two-sample t-test (see appendix 5). There was no significant difference between MNga2 and CM2177-2 at days 8 and 15 (appendix 5). However by 27dpi CM2177-2 was showing significantly (P= 0.014 t-test) less symptoms than MNga2.

It can be tentatively stated that when infiltrated with $1x10^6$ cfu ml^{-1} MCol22 is susceptible to *Xam* strain I56 whereas MNga2 and CM 2177-2 both have some degree of resistance, with CM2177-2 slightly more resistant.

Lobes infiltrated with $1x10^4$ cfu ml^{-1} showed less rapid symptom development than lobes infiltrated with $1x10^6$ cfu ml^{-1} (figure 3.2). MCol22 exhibited significantly more symptoms than MNga2 and CM 2177-2 at 15 and 22 dpi and significantly more symptoms than CM 2177-2 at 27 dpi (appendix 5). Whilst MNga2 had a higher mean SI than CM 2177-2 at 15, 22 and 27 dpi the differences were not significant (appendix 5). Again for lobes infiltrated with $1x10^4$ cfu ml^{-1} the ranking order appears to be the same as for lobes infiltrated with the higher inoculum level (CM2177-2 is slightly more resistant than MNga2 which is more resistant than MCol22).

Petiole stabbing proved to be the most variable method of inoculation. The mean standard deviation of SI following petiole stabs was 0.63 compared with 0.39 and 0.34 for leaf infiltration with $1x10^6$ and $1x10^4$ cfu ml^{-1} respectively. Because of this high variability few of the differences seen between cultivars (figure 3.3) are significant when tested with the Mann-Whitney U test (appendix 5). However a tentative susceptibility scale would be MCol22 as the most susceptible, then CM 2177-2 and MNga2 as the most resistant.

No symptoms were seen on any leaf sham-inoculated with 10mM $MgSO_4 \cdot 7H_2O$.

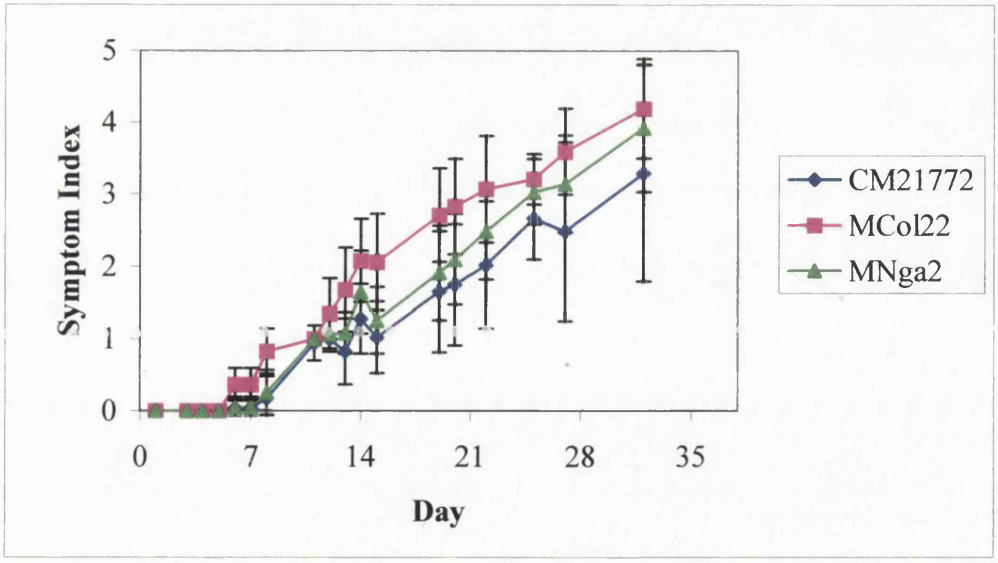


Figure 3.1 Disease symptoms caused by infiltration with $1x10^6$ cfu ml^{-1} Xam
A total of 16 CM 2177-2, 15 MCol22 and 16 MNga2 plants had two leaves infiltrated with a suspension of $1x10^6$ cfu ml^{-1} *Xam* strain I56. Each leaf was infiltrated three times. The symptom index (where 0 = No symptoms, 1= water-soaking, 2=chlorosis, 3=spreading chlorosis, 4= wilt of infected lobes and 5= wilt of whole leaf (see figure 2.2 for a picture of the different stages of infection)) was recorded for the whole leaf. Disease progression was monitored until 32 days post infection (dpi). MCol22 appears to be the most susceptible cultivar to leaf infiltrated *Xam* I56. Though none of the cultivars show high levels of resistance to CBB CM 2177-2 appears to be the most resistant to strain I56. Error bars = ± standard deviation. Data obtained with the help of Alan Bryant

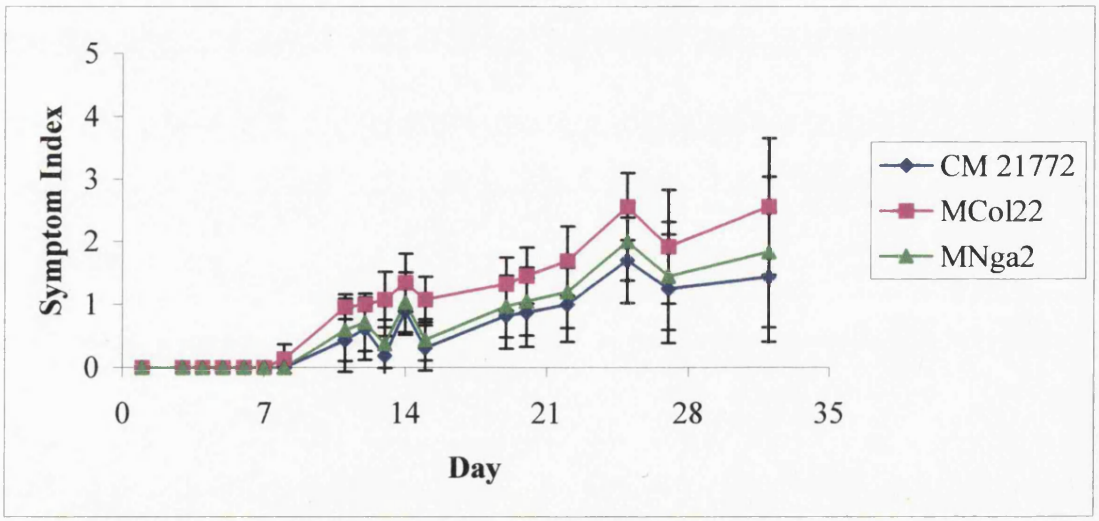


Figure 3.2 Disease symptoms caused by infiltration with $1x10^4$ cfu ml^{-1} Xam
See legend from figure 1 for details of inoculation. MCol22 appears to be the most susceptible cultivar to leaf infiltrated *Xam* I56. Though none of the cultivars show high levels of resistance to CBB CM 2177-2 appears to be the most resistant to strain I56. Error bars = ± standard deviation. Data obtained with the help of Alan Byrant

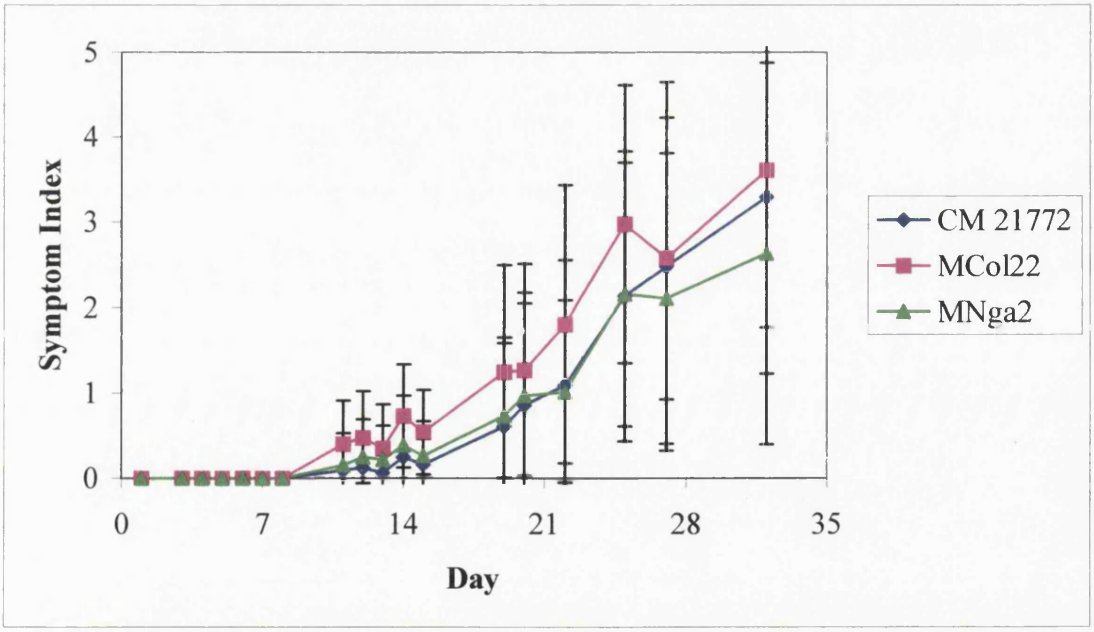


Figure 3.3 Disease symptoms caused by petiole stabbing with $1x10^6$ cfu.ml^{-1} Xam
A total of 16 CM 2177-2, 15 MCol22 and 16 MNga2 plants had two petioles injected with a suspension of $1x10^6$ cfu ml^{-1} *Xam* strain I56. The symptom index (where 0 = no symptoms, 1 = ooze exuding from wound, 2 = ooze spreading from initial site of infection, 3 = necrosis round infection site, 4 = wilt of individual lobes and 5 = wilt of whole leaf) was recorded. Disease progression was monitored until 32 dpi. MCol22 appears to be the most susceptible cultivar to petiole stab, however in contrast to leaf infiltration MNga2 appears to be more resistant than CM 2177-2. Error bars = ± standard deviation. Data obtained with the help of Alan Bryant.

3.4 Discussion

The data obtained from this study indicates that CM 2177-2 is the most resistant of the three cultivars to *Xam* infiltrated into the leaves. However MNga2 appears to be the most resistant cultivar to *Xam* introduced *via* a petiole stab. MCol22 was the most susceptible cultivar in this study. *Xam* strain I56 was virulent on all three cultivars indicating that it is a suitable strain with which to study pathogenicity.

The most resistant cultivar in this study was dependent on the inoculation method used. A similar effect had been noted previously where CM 2177-2 was more susceptible than MNga2 and MCol22 to *Xam* introduced by stem stabbing but more resistant than MNga2 and MCol22 to *Xam* introduced *via* leaf wounds (Restrepo *et al.*, 2000a). Different inoculation techniques mimic different infection methods likely to occur in nature. Leaf infiltration mimics natural infection through stomates and petiole-stabbing mimics direct entry into the xylem through wounds. Resistance of cassava to *Xam* is polygenic and it is likely that resistance acts during different stages of infection. In recent studies attempts have been made to map resistance of cassava to *Xam* using MNga2 and CM 2177-2 and their progeny in both glasshouse and field trials (Jorge *et al.*, 2000; Jorge *et al.*, 2001). One QTL was conserved between the glasshouse and field trials and is suspected to be involved in the resistance phenotypes ingressed from *M. glaziovii* into MNga2 (Jorge *et al.*, 2001). As MNga2 appears to be more resistant than CM 2177-2 to xylem-introduced bacteria (this study and table 3.1) it is possible that the resistance ingressed from *M. glaziovii* acts at the level of xylem cells. Mechanisms of cassava xylem resistance are likely to include tyloses, gel formation and production of antimicrobials (Kpémoua *et al.*, 1996). However, field trials have tended to show CM 2177-2 as more resistant than MNga2 (table 3.1). *Xam* can enter cassava *via* stomates and this may be the most common source of natural infection. In this case foliar resistance mechanisms would be more effective in stopping the spread of *Xam*. It appears that CM 2177-2 is more resistant to foliar infection than MNga2, which could explain why CM 2177-2 tends to be more resistant than MNga2 in field trials. Foliar resistance mechanisms to *Xam* are unknown in cassava. In CM 2177-2 they probably act after *Xam* has entered the plant as resistance was seen when bacteria were forced into the leaf. The assertion that resistance is only manifest in the xylem of cassava plants (Kpémoua *et al.*, 1996) appears to be incorrect.

Recently, resistance of cassava to *Xam* has been shown to be dependent on the strain of *Xam* used (Verdier *et al.*, 1998, Restrepo *et al.*, 2000a). In this study a highly virulent strain of *Xam* isolated from Nigeria was used (K. Wydra personal communication). A more accurate picture of the resistance of the cultivars could be obtained by using more strains. However, African isolates of *Xam* are generally less diverse than South American isolates

(Verdier *et al.,* 1993) and it is likely that strain I56 is representative of *Xam* encountered by cassava growing in Africa.

Resistance to CBB is also dependent on the conditions of cassava growth. A cassava plant may be resistant to *Xam* when grown in one ECZ but susceptible in another ECZ (Restrepo *et al.*, 2000). The three lines used in this study have been selected to grow under different conditions. MCol22 and MNga2 are adapted to ECZ 1 (sub-humid tropics), which is markedly different to ECZ 2 (acid-soil savannas) for which CM 2177-2 is adapted. The conditions of the glasshouse during this study were inevitably different to both of these ECZs; the low natural light levels in Europe during October compared to the tropics is one likely disparity although supplementary illumination was used. However the temperature range was suitable and humidity was kept high throughout. Symptoms continued to develop and were typical of that observed under field conditions in Benin (author's observations) and in Colombia and Togo (R Cooper, personal communication). The only difference was that systemic infection did not occur during the trial.

In the light of this study and others (table 3.1) it appears that the two parents of the cassava molecular genetic map are not ideal to elucidate components of resistance to CBB. If a molecular genetic approach was to be undertaken, two cultivars with highly divergent reactions to *Xam* in addition to high genome polymorphisms should be selected to create a new mapping population. Wild species of *Manihot* (the progenitors of cassava) may contain the variance needed (Nassar, 2000).

Chapter 4

Pathogenicity of *Xanthomonas axonopodis* pv. *manihotis*

4.1 Introduction

To date, no unambiguous pathogenicity or virulence factors have been reported for *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). A pathogenicity gene, termed *pthB*, with similarity to the *avrBs3* family of effector genes was reported to be present in all pathogenic strains of *Xam* (Verdier *et al.,* 1998). But no biochemical data have been presented on this gene and strains of *Xam* lacking only *pthB* have yet to be created and characterised. Extracellular polysaccharide (EPS) from *Xam* has been localised in the intercellular spaces of infected cassava plants using antibodies specific to EPS from *X. campestris* pv. *campestris* (Boher *et al.,* 1997) and in this laboratory detected ultrastructurally in infected xylem (Deshappriya, 1992). Whilst an EPS minus mutant of *Xam* has been claimed (Boher *et al.,* 1995), no characterisation or pathogenicity studies have been reported. Ultrastructural studies of *Xam* infected cassava plants revealed degradation of cassava cell walls and release of pectin fragments (Boher *et al.,* 1995). Pectolytic enzymes have been reported from a number of xanthomonads (Rudolph, 1993) though no clear role in pathogenicity has been established. Pectate lyase (Pel) activity was detected from some strains of *Xam* grown on cassava cell walls and unusually, the activity appeared to comprise a single high pI isoform (Deshappriya, 1992). A toxin, 3-methylthiopropionic acid, was hypothesised to be responsible for some of the symptoms of CBB (Perreaux *et al.,* 1982) but recent data has shown that this is not the case (Day, 2001). In view of the previous evidence and the practicality of performing genetic analysis, for this study EPS and Pel were investigated as two potential pathogenicity factors.

The most effective way to show that a potential pathogenicity or virulence factor functions as such *in planta* is to construct pathogen strains with defined mutations and test for altered pathogenicity. In xanthomonads mutations can either be introduced randomly with transposons (Turner *et al.,* 1984) or targeted to a particular gene using marker exchange (e.g. Slater *et al.,* 2000). However, no attempt has previously been made to disrupt genes from *Xam* either in a random or targeted manner. EPS production is a suitable target for gene disruption because the biosynthesis of EPS from *X. c.* pv. *campestris* has been described in detail (Katzen *et al.*, 1998). The *gum* gene cluster involved in the production of EPS appears to be well conserved both in other xanthomonads (Dharmapuri and Sonti, 1999) and in *Xylella fastidiosa* (da Silva *et al.,* 2001). It is therefore likely that the *gum* cluster will be conserved in *Xam*. Studies on the pathogenicity of *X. c.* pv. *campestris* strains with a single gene missing (*gumD*) that oblates xanthan production showed reduced virulence (Chou *et al.,* 1997; Katzen *et al.,* 1998). Transposon insertions into *gumG* from

X. oryzae pv. *oryzae* also disrupted EPS production and resulted in a loss of virulence (Dharmapuri and Sonti, 1999).

In contrast to the role of EPS, *pel* genes have failed to reveal a virulence function (Liao *et al.*, 1996). However Pel was the only extracellular factor found from *Xam* that killed cassava suspension cells (Deshappriya, 1992). Isoelectric focusing of enzymes secreted from *Xam* grown on host cell walls indicated a single isozyme of Pel (Deshappriya, 1992), making it an ideal target for gene disruption.

Genetic manipulation of xanthomonads is difficult, mainly because of the presence of restriction-modification systems (Daniels and Leach, 1993). A method of transforming *Xam* has been reported using electroporation where broad host range plasmids of three different sizes (51, 9.3 and 3.3 kb) were electroporated in *Xam*. A field strength of 14 kV/cm and a DNA concentration of 30 ng plasmid /sample gave a good transformation efficiency (Ferreira *et al.*, 1996).

The pKmob series of plasmids enables gene disruption experiments to be conducted in a wide range of bacteria (Schäfer *et al.*, 1994). The plasmids can be transferred to a range of bacteria *via* conjugation and are based on the replicon pMB1, so are only maintained in *E. coli* and closely related species of the genera *Salmonella* and *Serratia* (Schäfer *et al.*, 1994). In addition they have a multiple cloning site, kanamycin selection and the pK*mobsacB plasmids have a modified *sacB* gene, which confers sucrose sensitivity to Gram negative bacteria (Schäfer *et al.*, 1994). In order to create a defined, marker-free, deletion in a target gene, the gene of interest is cloned into pK*mobsacB. Restriction digests then remove a section of the target gene to give a truncated version. The resulting knockout vector is then introduced into the bacteria. Because the plasmid cannot be maintained, any antibiotic resistant colonies will result from homologous recombination between the target gene in the bacteria and the truncated form of the gene present in the knockout vector. The bacteria will now have two forms of the target gene, a full-length version and a truncated version separated on the chromosome by the vector DNA. Excision of the plasmid is selected for by growing cells on 10% sucrose. The *sacB* gene causes sucrose to be toxic to bacteria and any resulting colonies will have lost the plasmid and a copy of the gene of interest. Either the wild type gene or the truncated form will be the only copy left in the chromosome (Schäfer *et al.*, 1994) (figure 4.1). In *Corynebacterium glutamicum* the proportion of mutant to wild type colonies resulting from plasmid excision is approximately 55:45 (Schäfer *et al.*, 1994). In *X. c.* pv. *campestris* the proportion varies between 10% and 0.5% mutants depending on the gene investigated (M. Dow personal communication). It is possible to put an antibiotic resistance gene in the truncated version

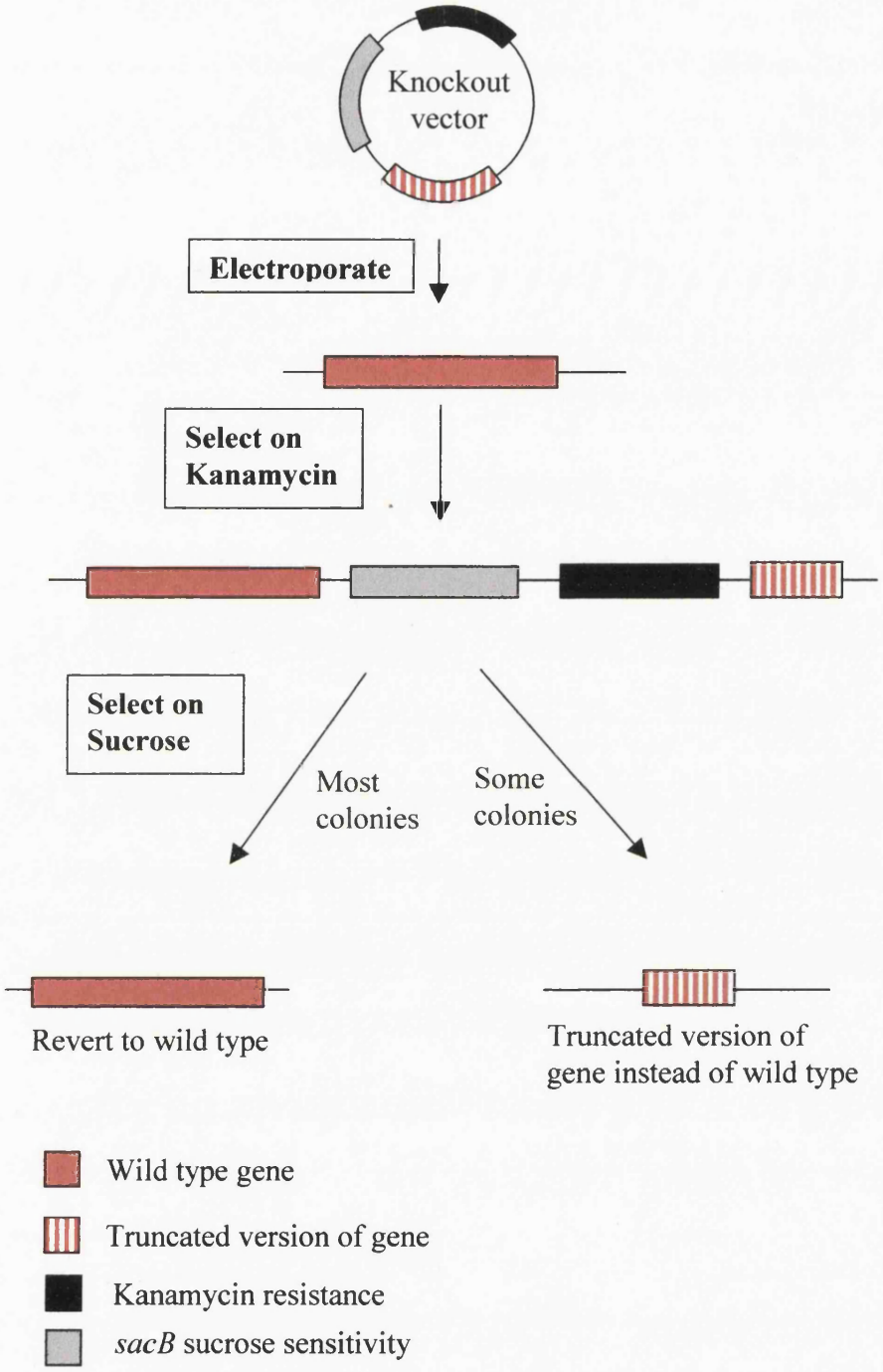


Figure 4.1 Gene replacement using the pK*mobSacB plasmids. A truncated version of the gene of interest is cloned into the pK*mobSacB plasmid and electroporated into xanthomonas. Because pK*mobSacB is not maintained in xanthomonads any resulting kanamycin resistant colonies result from integration of the plasmid into the genome *via* homologous recombination with the wild type gene. By selecting on sucrose rare double cross over events can be detected where the plasmid is lost from the genome leaving either the wild type version of the gene or the truncated version. Figure adapted from Schäfer *et al.*, 1994.

of the target gene in order to aid selection of mutant strains (e.g. Slater *et al.*, 2000), however marker-free deletions are preferable for pathogenicity studies (M. Dow personal communication)

To determine the role(s) of EPS and pectate lyase in pathogenicity, attempts were made to construct *Xam* strains with defined mutations in specific genes. Initially homologues of *gumD* and *pelA* were to be isolated followed by construction of mutant strains and assessment of pathogenicity and virulence.

4.2 Materials and Methods

4.2.1 Isolation of genomic DNA from *Xam* for PCR

The method used for DNA extraction for PCR was essentially as previously described (Boucher *et* al., 1985). A single colony was transferred to 5ml of NYGB and grown overnight at 30°C with shaking at 200rpm. 1.5ml of overnight culture was removed to a microfuge tube and centrifuged at 13,000g for 2 min. The resulting pellet was washed twice in Tris-EDTA buffer (50mM Tris/HCl, 20mM EDTA, pH8) and resuspended in 900µl Tris-EDTA buffer. Cells were lysed by the addition of 100µl 10% SDS and incubation for 5 min at 65°C. The lysate was deproteinated by the addition of an equal volume of phenol saturated in TE (10mM Tris, 1mM EDTA, pH 8.0) mixing and centrifuging at 13,000g for 5 min. The supernatant was removed to a fresh tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 (v/v/v)) was added, mixed with the supernatant and centrifuged as before. 750µl of the aqueous phase was removed to a fresh tube and DNA precipitated with the addition of 75µl 3M sodium acetate (pH 5.2) and 750µl 100% ethanol. DNA was collected by centrifugation at 13,000 rpm for 15 min and washed in 70% ethanol, air dried and resuspended gently in TE buffer. RNA was removed with the addition of 1µl 10mg ml^{-1} RNase A (Sigma-Aldrich) made DNase free according to Sambrook *et al.* (1989) and incubating at room temperature for 10 min. DNA prepared by this method was adequate for PCR but not suitable for restriction digests.

4.2.2 Isolation of high quality genomic DNA

The method for extracting large quantities of genomic DNA was essentially as detailed in Ausubel *et al.* (1990). Cells from a 100ml overnight culture were pelleted by centrifugation at 4000g for 10 min. Cells were resuspended in 9.5ml TE buffer and lysed with the addition of 0.5ml 10% SDS. Proteins were degraded with 50µl of 20 mg ml^{-1} proteinase K (Sigma-Aldrich) incubated at 37°C for 1 h. Polysaccharides were precipitated by addition of 1.8ml 5M NaCl and 1.5ml CTAB/NaCl solution (10% CTAB in 0.7M NaCl) and incubation at 65°C for 20 min. Proteins and precipitated polysaccharides were removed by the addition of an equal volume of chloroform/isoamyl alcohol (24:1 (v/v)) mixed thoroughly and centrifuged at 6000g for 10 min to separate phases. The aqueous phase was removed to a fresh tube and DNA precipitated with 0.6 volumes of propan-2-ol. DNA was collected on a hooked Pasteur pipette (created by heating the end of the pipette over a Bunsen burner flame) and washed in 70% ethanol. Washed DNA was dissolved in 4ml TE buffer overnight at 4°C, then the concentration of DNA estimated with a spectrophotometer and adjusted to 100µg ml^{-1}. 1.075 g CsCl and 50µl of 10mg ml^{-1} ethidium bromide were added per ml of DNA solution. The resulting solution was

subjected to ultracentrifugation as described by Sambrook *et al.* (1989). The resulting DNA band was removed using a wide-bored needle. Ethidium bromide and CsCl were removed by extraction with water-saturated butanol and dialysis respectively (Sambrook *et al.*, 1989).

4.2.3 Southern Blot Analysis

0.5 μg of *Xam* genomic DNA was digested to completion with a number of restriction endonucleases as manufacturer's instructions (New England Biolabs). The digests were separated on a 0.7% agarose TBE gel at 30v overnight. Gels were viewed under UV illumination before the DNA was alkali transferred to Hybond N+ (Amersham Biosciences) as manufacturer's instructions.

4.2.4 PCR amplification of fragments of gumD and pelA

Primers were designed to amplify a 569bp fragment of gumD from *X. c.* pv. *campestris* (see appendix 3). These primers were used to amplify a section of *Xam* strain I56 genomic DNA. 5ng genomic DNA was used as a template in 50μl PCR reactions containing: 50 pmol of gumD5' (see appendix 3) 50 pmol gumD3' (see appendix 3) 1 unit Amplitaq Gold DNA polymerase (Applied Biosystems, Warrington, UK), 1x Amplitaq buffer, 200μM dNTPs (Promega) and 1mM $MgCl_2$. The following PCR conditions were used: 10 min denaturation at 95°C followed by 40 cycles of 1 min denaturation at 95°C, 30 s annealing at 52°C and 45 s extension at 72°C with a final soak at 72°C for 5 min.

Five *pelA* sequences from both xanthomonads and pseudomonads were aligned using ClustalW. Primers were designed against conserved regions to amplify a 430bp fragment of *pelA* (see appendix 3). Amplification conditions were the same as above.

Amplification products were purified using the Qiaquick gel extraction kit (Qiagen, Crawley, West Sussex, UK) and 5ng sequenced using an ABI Prism 377 automated sequencer (Applied Biosystems).

4.2.5 Cloning of gumD

A partial genomic library of *Xam* I56 DNA digested to completion with *Eco* RI, was created in the Uni-ZAP XR λ vector (Stratagene) as described in chapter 2. The library was screened for a *Xam* gumD homologue using the PCR isolated gumD fragment.

4.2.6 Creation of pGX2

pGUM (pBluescript containing a 5kb *Eco* RI fragment containing all of *gumD*) was digested with *Nco* I (New England Biolabs) to release a 1kb fragment of gumD. The

plasmid was religated to create pGX. The truncated gum fragment was released from pGX with an *Eco* RI digestion and ligated into pK18mobsacB (Schäfer *et al.*, 1994) (provided by Dr. J. Kalinowski, University of Bilefeld, Germany) to create pGX2.

4.2.7 Transformation of *Xam*

Electrocompetent *Xam* cells were made following an adapted protocol for *X. c.* pv *campestris* (Wang and Tseng, 1992). Fifty ml NYGB was inoculated with a single colony of *Xam* I56 and grown at 28°C for 24 h. One ml of this 24 h culture (*ca.* $2x10^9$ cfu) was transferred to 200ml NYGB and grown at 28°C with shaking until the optical density at 600nm reached 0.3 (*ca.* 6 h).

Cells were harvested by centrifugation at 3,000g and washed four times with 40ml ice-cold 10% glycerol (pH 7.0) to remove salts that interfere with the electroporation procedure. Cells were then resuspended in 400µl ice-cold 10% glycerol and snap frozen on dry ice in 50µl aliquots. Electrocompetent cells were stored at –70°C.

Transformation was carried out with a Bio-Rad gene pulser electroporator. Fifty µl competent cells were thawed on ice and transferred to a pre-chilled electroporation cuvette (0.2cm gap, Bio-Rad). DNA dissolved in no more that 5µl MilliQ water (between 50-200ng for broad host range plasmids, 0.5-5µg for knockout vectors) was added to the cuvette and electroporation performed under the following conditions: field strength 2.5kV, capacitance 25µF and resistance 400Ω. One ml NYGB was added to the pulsed cells and they were transferred to a sterile microfuge tube before incubation at 28°C for 1h to allow expression of antibiotic resistance genes. Cells were harvested by centrifugation in a microfuge (3000g for 5 min) and lawned onto NYGA supplemented with appropriate antibiotics.

4.2.8 Quantification of EPS

Single colonies of *Xam* were grown for 7 d in 50ml NYGB. Cultures were centrifuged for 60 min at 5000g and the supernatant adjusted to 1M NaCl. Polysaccharides were precipitated by the addition of 2 vol of ethanol to the adjusted supernatant and shaking vigorously. Precipitation occurred immediately but was left at room temperature for several h. Filter papers (Whatman International, Maidstone, UK) were dried at 80°C until constant weight (*ca.* 2 h) and used to collect the EPS precipitate. The filters with the EPS were then dried at 80°C to constant weight and the difference between the two weights used to estimate the production of EPS per ml culture. At least three replicates were used.

4.2.9 Pectate lyase enzyme assay

Cell wall degrading enzyme production was induced over five days growth on minimal medium (see appendix 1) with cassava cell walls as the sole carbon source (Deshappriya, 1992). To assay for activity 0.5ml of *Xam* culture were removed aseptically, the sample centrifuged at 10,000g for 10 min in a microfuge and filtered through 0.45nm Millex syringe driven sterile filters (Millipore, Watford, UK) to obtain cell free culture fluids.

The pectate lyase assay consisted of 0.7ml buffered substrate (0.25% w/v polygalacturonic acid, 50mM Tris, 1mM $CaCl_2$, pH 9.0) with 0.3ml culture supernatant in a quartz cuvette. Pectate lyase activity was measured as an increase in absorbance at 235nm. Using the molar extinction coefficient of the unsaturated bonds produced (Nagel and Anderson, 1965) the change in optical density could be expressed as μmol unsaturated galacturonic acid produced per min.

4.3 Results

4.3.1 Genomic DNA extraction

Published genomic DNA extraction methods for xanthomonads provided DNA of sufficient quality for PCR. However attempts to isolate high quality genomic DNA for restriction digests from *Xam* proved to be difficult. Even after extensive phenol and chloroform extractions, contaminating macromolecules that inhibited restriction enzymes were present in genomic DNA. The use of a genomic DNA extraction kit (Qiagen) also resulted in DNA resistant to digestion. Only by the use of a caesium chloride gradient was DNA obtained of sufficient quality for restriction digestion.

4.3.2 Creation of an EPS minus strain of *Xam*

Primers designed against the gumD gene from *X. campestris* pv. *campestris* used to amplify *Xam* DNA resulted in a PCR product of approximately 500bp (figure 4.2). The sequence of this PCR product revealed over 80% nucleotide identity with the published sequence of *gumD* from *X. c.* pv. *campestris.* Southern blot analysis revealed that the gene was present as a single copy, which is essential for successful gene replacement. Southern analysis also revealed that the gumD homologue was present on an *Eco* RI fragment of *ca.* 5kb (figure 4.3).

In order to obtain a full-length gumD clone a partial genomic library of *Xam* strain I56 was constructed in Uni-ZAP XR λ (Stratagene). The advantage of using a small capacity excisable cloning vector such as Uni-ZAP XR λ is that it minimises downstream subcloning. The main disadvantage is that a complete genomic library is not obtained. *Xam* DNA was digested to completion with *Eco* RI and agarose gel analysis indicated that the average size was *ca.* 6kb. To obtain an equimolar ratio of insert to vector 0.15μg, digested *Xam* DNA was ligated into 1μg of Uni-ZAP XR λ vector (41kb) pre-digested with *Eco* RI. The ligation reaction was packaged and gave a primary library of $1.3x10^5$ pfu with a background (vector with no insert) of 15%. *Xam* has a genome size of approximately 5 Mb and $1.3x10^5$ pfu with an average insert size of 6 Kb gives over 100 genome equivalents even when the high background is taken into account. After amplification the secondary library had a titre of $1x10^{11}$ $pfu.ml^{-1}$. Four plates of $5x10^4$ pfu (each containing *ca.* 50 genome equivalents) were screened for gumD-containing phage. A total of 26 positive plaques were found over the four plates; this was less than the expected 200, indicating that the average insert size may have been lower than 6 kb or that smaller fragments were more readily ligated into the vector. Sixteen positive plaques were picked and subjected to a second round of screening resulting in selection of six plaques for plasmid excision using

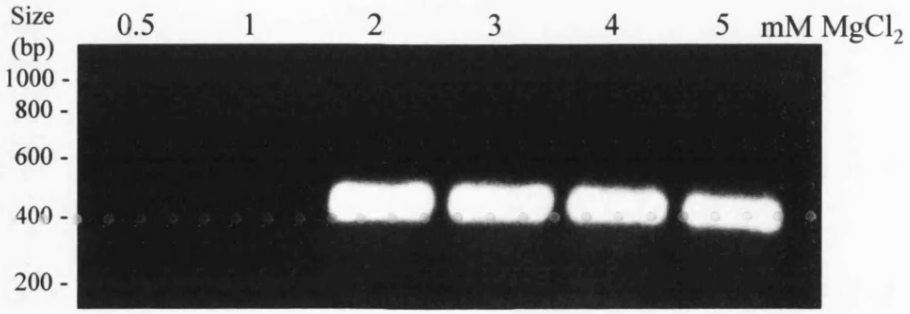


Figure 4.2 Amplification of a 500bp fragment of *gumD* from *Xam*
Primers designed against *gumD* from *X. c* pv. *campestris* were used on genomic DNA isolated from *Xam* strain I56. Increasing levels of $MgCl_2$ were used as initial PCR with 1.5mM $MgCl_2$ failed to yield any product (final concentration is indicated at the top of the gel). Molecular weight is shown on the left hand side of the gel.

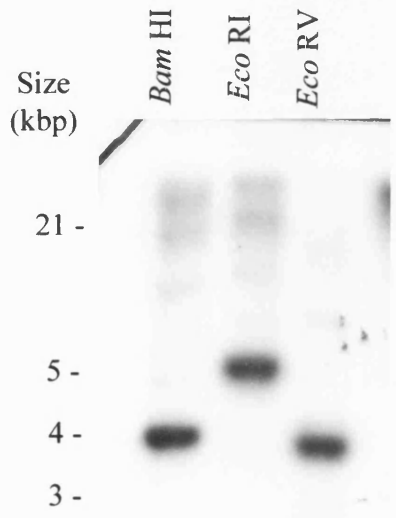


Figure 4.3 Southern blot of *Xam* I56 genomic DNA probed with 500bp of *gumD*.
DNA was restricted with the enzymes indicated and fractionated on a 0.8% agarose TBE gel before transfer to Hybond N+ membrane. The 500bp of *gumD* had previously been isolated from *Xam* using PCR primers designed against *X. campestris* pv. *campestris gumD*. Size of molecular weight standards are indicated on the left hand side of the gel. This blot indicates that *gumD* is a single copy gene in *Xam*. Molecular weight is shown on the left hand side of the gel.

the ExAssist helper phage. Colony PCR using gumD primers was then used to select colonies for further study. The plasmid containing the gumD homologue was called pGum and restriction digests revealed it contained around 5 Kb of genomic DNA inserted at the *Eco* RI site. Because of the relatively large size of the insert and the high copy number of pBluescript *E.coli* containing pGUM were grown at 30°C instead of 37°C to avoid stressing the cells.

The insert in pGum was partially sequenced and shown to contain all of gumD along with parts of gumC and gumE, indicating a similar arrangement to that found in *X. c.* pv. *campestris* and *Xy. fastidiosa*. Complete sequencing of gumD revealed it to be 1452 bp long and to encode a polypeptide of 54.3 kDa with significant similarity to the *gumD* genes from *X. c.* pv. *campestris* and *Xy. fastidiosa* (figure 4.4). *Xam* gumD contains two *Nco* I restriction sites (at 108 and 1063 bp). Restriction digests showed no other *Nco* I sites in pGUM (figure 4.5). In order to create a knockout vector pGUM was digested with *Nco* I to remove the majority of gumD and religated to create pGX. Restriction digests (figure 4.5) and PCR reactions (figure 4.7), showed the absence of gumD from pGX. pGX2 was then created by ligating the truncated gum genes into pK18mobsacB at the *Eco* RI site (figure 4.6). Gene replacement studies were then initiated using pGX2. Electrocompetent *Xam* cells transformed with 1μg of pGX2 yielded *ca.* 20 kanamycin resistant colonies. pGX2 will not be maintained in *Xam* so these colonies must have resulted from homologous recombination between pGX2 and the *Xam* genome.

Initial attempts to excise the plasmid from the genome were unsuccessful. Sucrose insensitive colonies resulting from plating directly onto 10% sucrose NYG agar were morphologically indistinguishable from wild type colonies. Further attempts were made by picking single colonies to 10% sucrose NYG broth growing for 24 h then lawning onto NYG agar. However all colonies were again indistinguishable from wild type. Finally 30 kanamycin resistant colonies were grown in NYG broth without selection for 24 h. Approximately 10^6 cells (100μl of a 1/100 dilution of an overnight culture) were then lawned onto 10% sucrose NYG agar. One of these plates resulted in colonies with two different morphologies. One form was the same as wild type whereas the other appeared to be less mucoid. Both colonies were present in approximately equal numbers on the plate.

Colony PCR using gumD primers failed to amplify any product from the less mucoid colonies but gave a band of the same size as *Xam* I56 with the mucoid colonies (figure 4.7). Genomic DNA proved to be easier to extract from mutants than from wild type *Xam* with a genomic DNA extraction kit (Qiagen) providing DNA of sufficiently high quality for restriction digests.

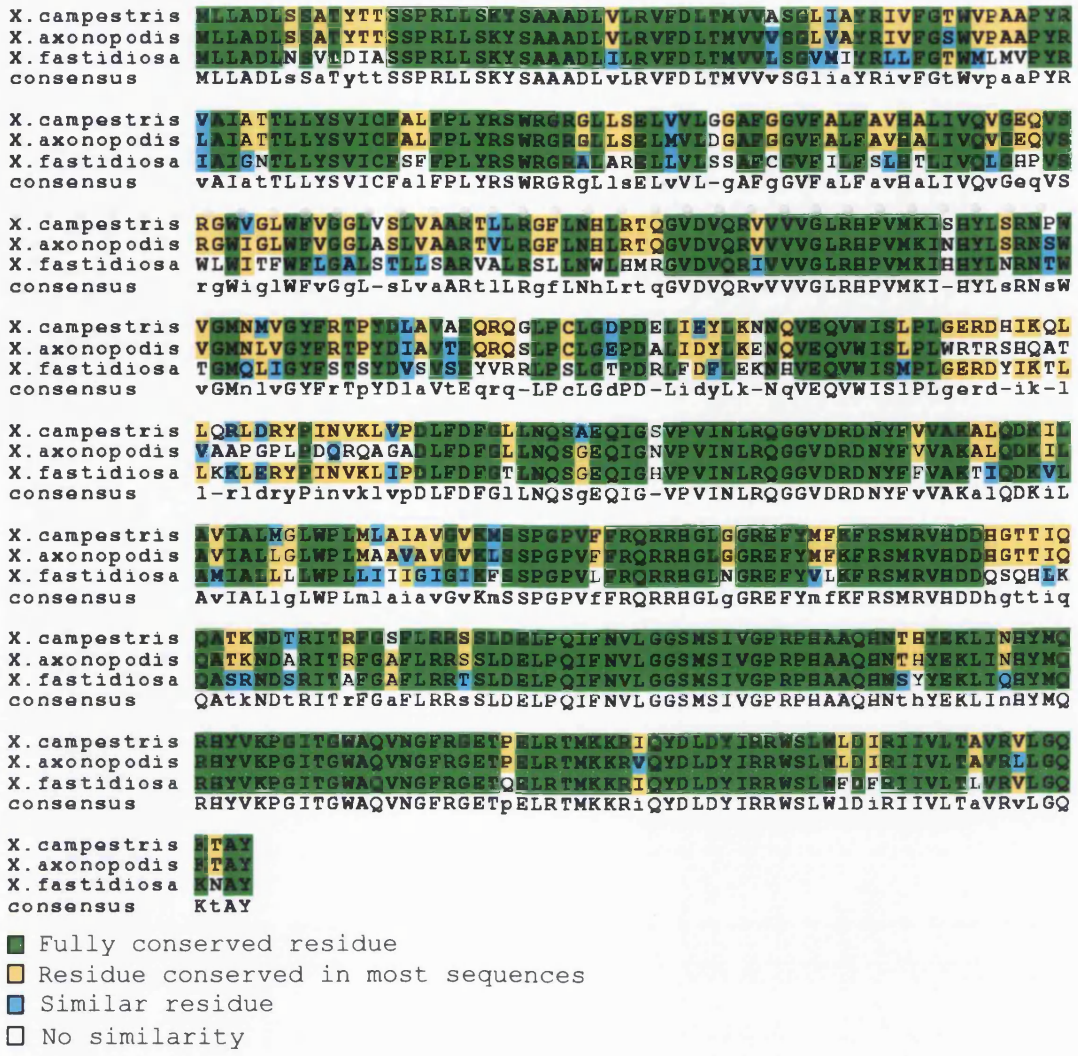


Figure 4.4 Alignment of *gumD* protein sequence from three phytopathogens
The protein sequence deduced from *gumD* genes of *X. c* pv. *campestris* (X. campestris) (Katzen *et al.*, 1998), *Xylella fastidiosa* (X. fastidiosa) (da Silva *et al.*, 2001) and *Xam* (X. axonopodis) (this study) were aligned using ClustalW (Thompson *et al.*, 1994). Fully conserved residues are shown in uppercase letters in the consensus sequence and partially conserved or similar residues are shown in lower case. The alignment shows the similarity of the three proteins are indicating that they probably have identical roles in the biosynthesis of extracellular polysaccharide.

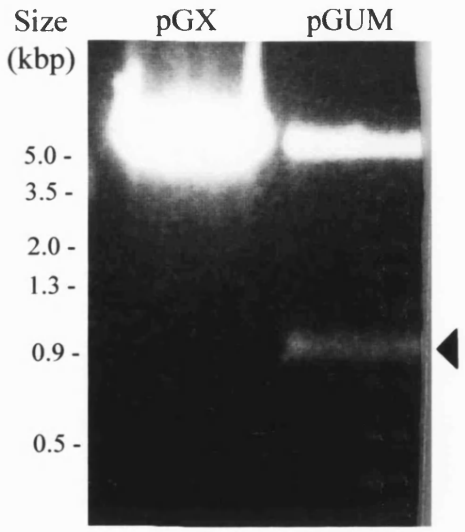


Figure 4.5 *Nco* I digestion of pGUM and pGX
When pGUM was digested to completion with *Nco* I a band of approximately 1kb (black arrowhead) was visible, indicating that the two *Nco* I sites identified by sequencing were present and unique. On the left hand side pGX digested to completion with *Nco* I can be seen; note the absence of a band at 1kb despite the large amount of DNA subjected to electrophoresis, indicating that pGX did contain a truncated version of *gumD.* Molecular weight is shown on the left hand side of the gel.

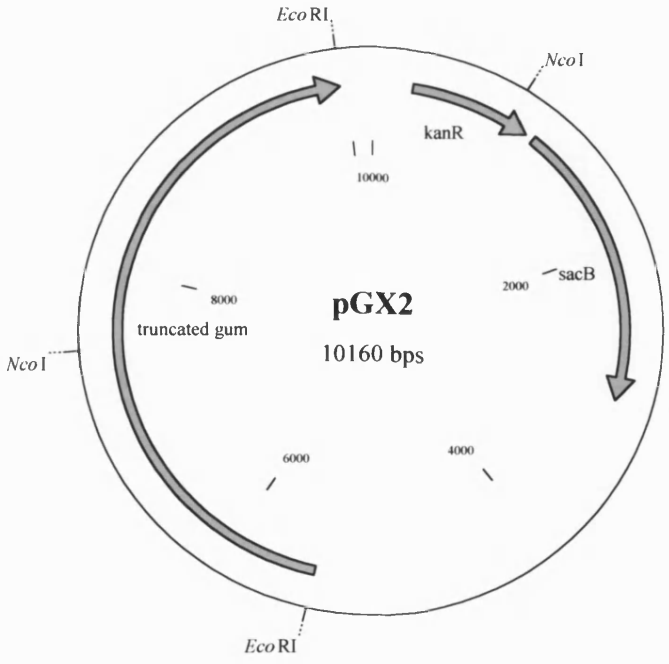


Figure 4.6 Plasmid map of pGX2 (used to create EPS$^-$ strains of *Xam)*
A 5.5kb *Eco* RI fragment of *Xam* genomic DNA containing all of *gumD* and parts of *gumC* and *gumE* was digested with *Nco* I to remove a 1kb section of *gumD.* This "truncated gum" DNA fragment was cloned into the knockout vector pK18*mobsacB* to create pGX2. kanR = kanamycin resistance cassette *sacB*= levansucrase, which is lethal for gram negative bacteria growing on sucrose. *Eco* RI and *Nco* I sites are indicated on the map.

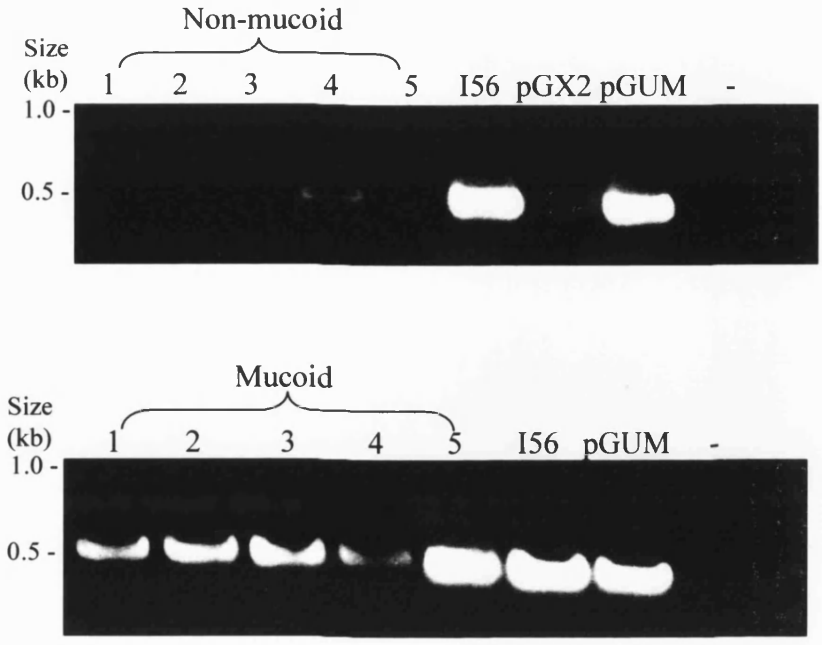


Figure 4.7 Colony PCR of mucoid and non-mucoid colonies resulting from excision of pGX2 from *Xam* I56

The upper panel shows PCR products from five non-mucoid colonies, the lower panel shows PCR products from five mucoid colonies on the same plate. A portion of a single colony was transferred to 50ml ddH_2O, heated to 100°C for 10 min and 10ml used as template for a PCR reaction. 10µl of PCR reaction was subjected to electrophoresis. All non-mucoid colonies failed to amplify a PCR product and all mucoid colonies gave a PCR product of the predicted size (though the amount of product is variable, probably due to the nature of genomic colony PCR). This indicated that, provided they were not due to contamination, non-mucoid colonies had lost *gumD*. Several control reactions were included; I56 = a colony of *Xam* I56 treated as colonies on the sucrose plate, pGX2 = the knockout plasmid to ensure the truncated version of *gumD* did not give a PCR product, pGUM = a positive control to ensure the colony PCR was working and - = no template. Molecular weight is shown on the left hand side of the gel.

Southern blots probed with the truncated gum fragment showed a band shift that could be explained by the loss of gumD (figure 4.8). These results indicate that the less mucoid colonies are *Xam* mutants with no gumD gene. Gravimetric assessment of the EPS production showed that mutant strains produced on average 0.2mg of ethanol precipitatable material per ml of culture, compared with 1.2mg for every ml of WT bacteria (figure 4.9).

4.3.3 Pathogenicity of EPS minus strains

Cassava plants of cultivar MCol22 (susceptible to CBB) were infected with either *Xam* I56 (WT) or *Xam* I56 *gumD* minus (EPS^-). Three different inoculation methods were used; leaf infiltration with $1x10^6$ cfu ml^{-1}, leaf infiltration with $1x10^4$ cfu ml^{-1} and petiole stab with $1x10^6$ cfu ml^{-1}. To ensure that effects on pathogenicity were associated with the loss of *gumD* an independent EPS deficient mutant (EPS^-_b) was used to infect plants with leaf infiltration at a concentration of $1x10^6$ cfu ml^{-1}.

Symptom progression was more rapid in leaves infected with WT than EPS^- strains for all three treatments (figure 4.10). No significant difference was found between EPS^- and EPS^-_b and all further investigations concentrated on strain EPS^-. Based on the 0-5 symptom index (SI) scale, leaves infiltrated with $1x10^6$ cfu ml^{-1} WT bacteria showed water-soaking after nine days (SI = 0.97± 0.11), chlorosis after 14 days (SI = 1.88±0.58), spreading chlorosis after 20 days (SI = 3.11± 0.41) and lobe wilt after 28 days (SI = 4.11± 0.79). EPS^- bacteria induced water-soaking after 12 days (all leaves showed a SI of 1) and chlorosis after 26 days (SI = 1.86± 0.33). However, with EPS^- and EPS^-_b mutant strains symptoms never progressed beyond the initial infection point during the trial; representative leaves from 28 days post infection are shown in figure 4.11.

Leaves infected with $1x10^4$ cfu ml^{-1} bacteria showed a similar pattern of disease progression to leaves infected with $1x10^6$ cfu ml^{-1} but symptoms appeared more slowly. WT bacteria caused water soaking after 13 days (SI = 0.91± 0.28), chlorosis after 21 days (2.2± 0.77) and spreading chlorosis after 28 days (SI= 3.00±1.26). Most leaves infected with $1x10^4$ cfu ml^{-1} EPS^- bacteria showed water-soaking at 15 days (SI = 0.83± 0.38) and chlorosis at 26 days (SI= 1.70± 0.45). Symptoms never progressed beyond localised chlorosis, and this did not occur in all leaves.

Petiole stab proved to be the most variable method of inoculation; the mean standard deviation for WT stab inoculations was 1.25 compared with 0.40 for $1x10^6$ cfu ml^{-1} and 0.47 for $1x10^4$ cfu ml^{-1} WT leaf infiltrations. However no petiole stabbed with EPS^- bacteria showed symptoms associated with CBB; in contrast, by the end of the trial the majority (*ca.* 90%) of WT-inoculated leaves showed severe wilting (figure 4.11).

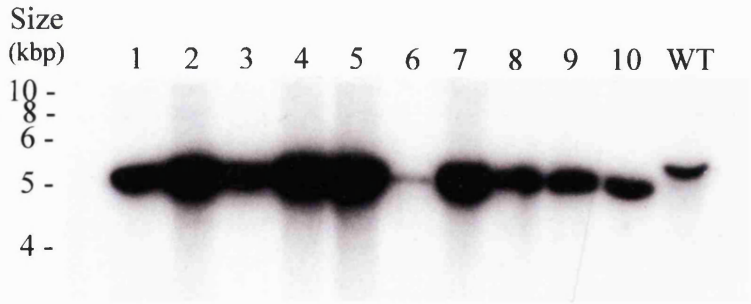


Figure 4.8 Southern blot of 10 independent *gumD* mutants and WT I56 probed with *Xam* DNA flanking *gumD*. Genomic DNA from 10 putative *gumD* mutants (lanes 1-10) and I56 (WT) was digested with *Eco* RI and fractionated on a 0.8% agarose TBE gel before transfer to Hybond N+. The blot was probed with the insert from pGX. All colonies selected showed a EPS$^-$ phenotype and all show a reduction in the size of the fragment hybridising to the pGX probe. Unequal loading makes quantification of the size difference difficult though a band shift is clear in all lanes compared with WT. Molecular weight standards are shown on the left hand side of the blot.

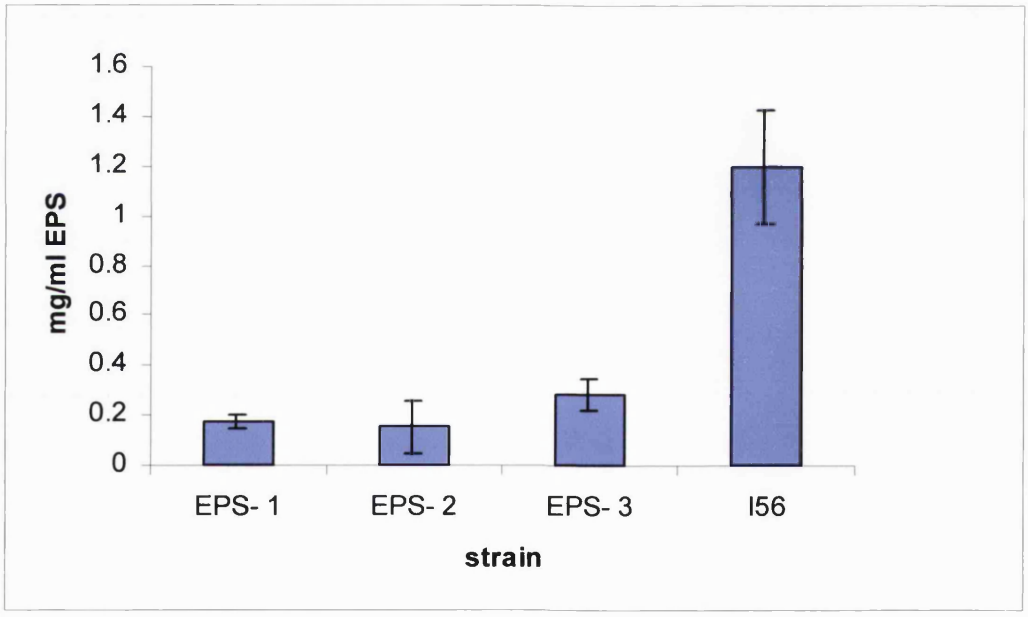


Figure 4.9 Quantification of EPS in the supernatant of WT and EPS$^-$ *Xam* cultures. Seven-day-old *Xam* cultures were centrifuged to remove bacteria and EPS in the supernatant precipitated with ethanol. The amount of precipitate was quantified gravimetrically. The three EPS$^-$ strains result from independent plasmid excision events and correspond to lanes 1, 2 and 3 in figure 4.9. EPS$^-$ strains produce significantly ($P = 0.016$, Mann-Whitney U test) less EPS than WT I56. Results are the mean of three separate cultures, error bars = ± standard error.

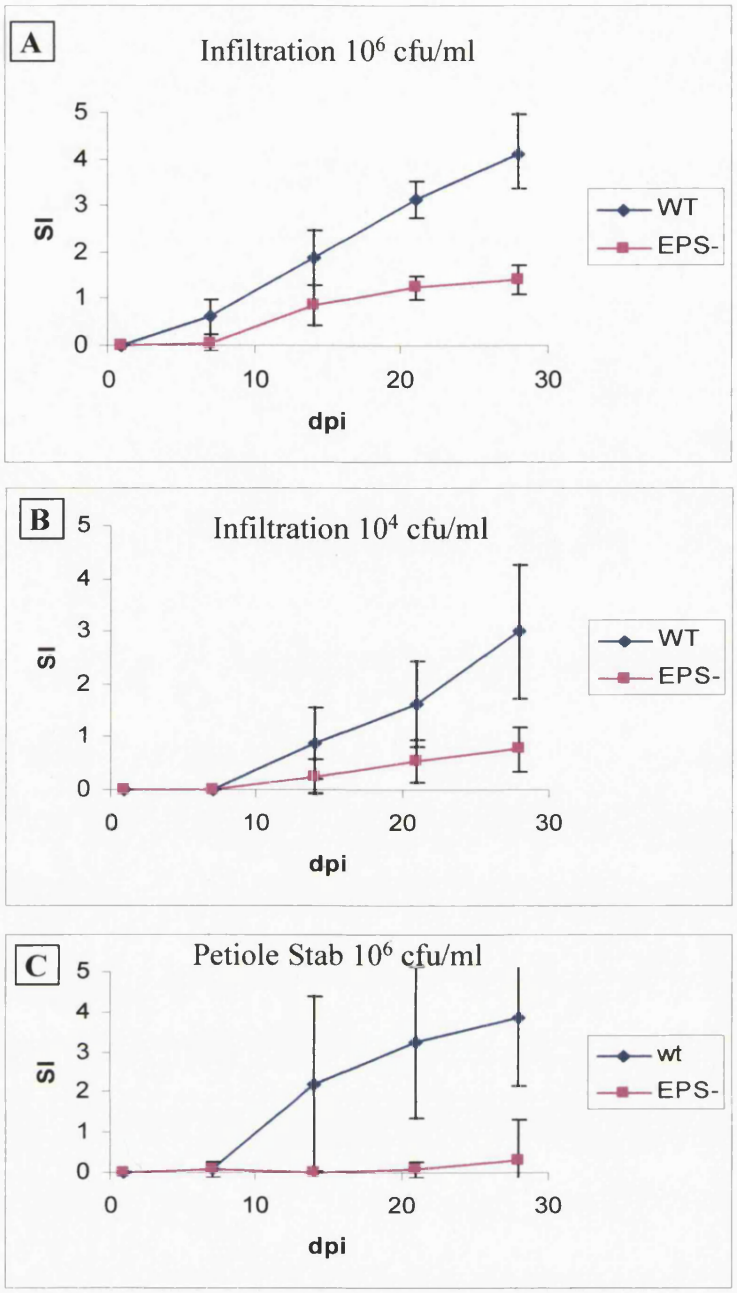


Figure 4.10 Symptom Index (SI) of MCol22 infected with WT and EPS$^-$ *Xam* by three methods.

A: SI progression of leaves infiltrated with 1x10^6 cfu ml^{-1} *Xam.* Two leaves from six plants were infiltrated with either WT or EPS$^-$ *Xam*. Symptom progression was recorded over a 4 week period. Plants infiltrated with EPS$^-$ bacteria show significantly slower symptom development than those infiltrated with WT bacteria.

B: SI progression of leaves infiltrated with 1x10^4 cfu ml^{-1} *Xam.* Legend as above

C: SI progression of petiole stabbed with 1x10^6 cfu ml^{-1} *Xam.* Two petioles from six plants were stabbed with either WT or EPS$^-$ *Xam*. Symptom progression was recorded over a 4 week period. Whilst plants infected with WT bacteria show symptom development, plants infected with EPS$^-$ *Xam* fail to show significant symptoms. Note the high variability of this method of inoculation.

dpi= days post inoculation, error bars = ± standard deviation. Data obtained with the help of Alan Bryant

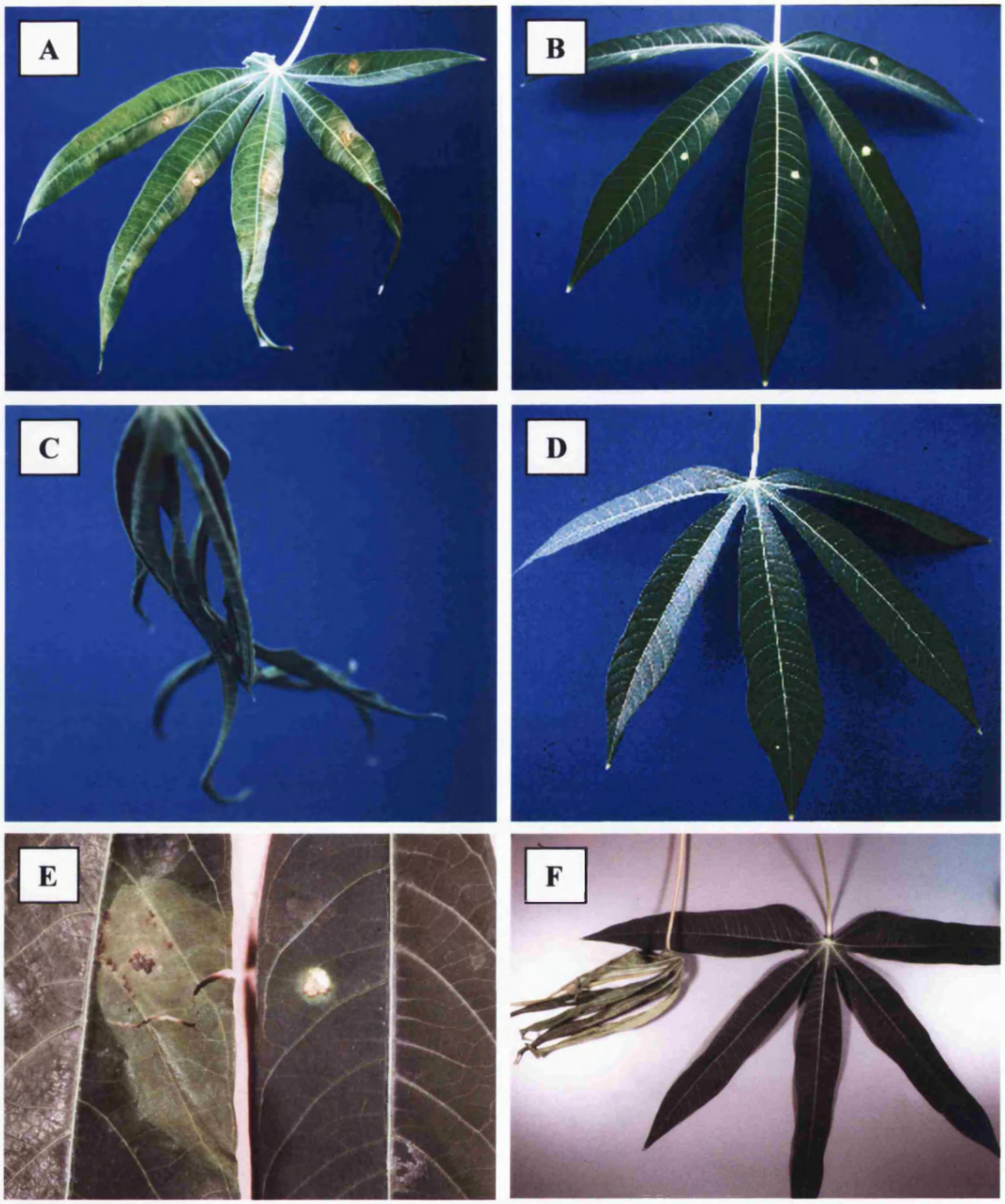


Figure 4.11 Pathogenicity of EPS$^-$ strain of *Xam* on cassava MCol22
Three inoculation methods were used to test the pathogenicity of EPS$^-$ as compared to WT *Xam*. Panels A and B show representative cassava leaves 28 days after syringe infiltration with a $1x10^6$ cfu ml^{-1} suspension of *Xam*. WT *Xam* (panel A) has caused extensive chlorosis and the lobes have begun to wilt, in comparison EPS$^-$ *Xam* (panel B) failed to cause symptoms away from the initial infection point. Panels C and D show representative cassava leaves 28 days after petiole stab with a $1x10^6$ cfu ml^{-1} suspension of *Xam.* WT *Xam* (panel C) has caused the whole leaf to wilt, in comparison cassava leaves infected with EPS$^-$ *Xam* (panel D) show no symptoms. Panel E shows a single infiltration point 32 days after infiltration with $1x10^4$ cfu ml^{-1}. WT *Xam* (left hand lobe) has caused extensive chlorosis and desiccation whereas EPS$^-$ *Xam* (right hand lobe) has only resulted in a small chlorotic halo round the initial infection point. Panel F shows leaves 32 days after petiole stab (as in panels C and D); again WT *Xam* (left) has induced significant disease whereas EPS$^-$ infected leaves show no symptoms. Data obtained with the help of Alan Bryant

Bacterial numbers were determined for leaves infiltrated with $1x10^6$ cfu ml^{-1}. At days 0, 2, 9 and 14 the numbers of bacteria in the initial infection point were determined and revealed that EPS$^-$ bacteria grew as well as WT bacteria (figure 4.12). After 14 d there was no significant difference in the number of EPS$^-$ bacteria compared with the number of WT bacteria at the site of infection (P= 0.31). Samples were later taken at distance from the main infection point 34 dpi. Very few (177±126 cfu cm^{-2}) EPS$^-$ bacteria were found 1 cm away from the initial infection point whereas large numbers of WT bacteria (1.68±0.36 x 10^6 cfu cm^{-2}) were found in the equivalent region. Numbers of bacteria per entire lobe were also determined and revealed that significantly (P= 0.009) more WT bacteria were present in the lobes (2.87±0.76 x 10^8 cfu cm^{-2}) than EPS$^-$ bacteria (1.64±1.06 x 10^6 cfu cm^{-2}). There was no significant (P=0.5) difference between the number of EPS$^-$ bacteria detected in a single infiltration point at 14 days and the number of EPS$^-$ bacteria present in a whole lobe at 34 days. There was a significant difference (P=0.001) between the number of WT bacteria in a single infection point at 14 days and the number of bacteria in a whole lobe at 34 days.

4.3.4 Possible role of pectate lyase in *Xam* pathogenicity

Three strains of *Xam* were incubated on minimal medium with cassava cell walls as a sole carbon source in order to induce cell wall degrading enzyme production. After 3 days incubation *Xam* strain 2967 showed significant pectate lyase activity. Strains 1222 and I56 showed little activity even after 7 d of incubation (figure 4.13) A preliminary pathogenicity test revealed that strain 2967 caused less severe symptoms on MCol22 when infiltrated than strain I56.

Of the 11 strains of *Xam* used in this study, four showed an amplification product of the predicted size (460bp) with *pelA* specific primers at an annealing temperature of 45°C (figure 4.14). Three of these PCR products (from strains 246, 1222 and 2507) were sequenced and showed *ca.* 90% identity to published *pelA* sequences from *X. c.* pv. *malvacearum* (figure 4.15). Using very pure genomic DNA, the *pelA* PCR product was also obtained from strain I56. A southern blot of genomic I56 DNA was probed with the I56 *pelA* PCR product (figure 4.16). *Xam* I56 *pelA* appears to be a single copy gene. Attempts were made to isolate the whole gene from the genomic library constructed to isolate *gumD*. However because of the large size of the *Eco* RI fragment *pelA* resides on (over the 10kb limit of Uni-ZAP XR λ) it proved impossible to isolate the gene.

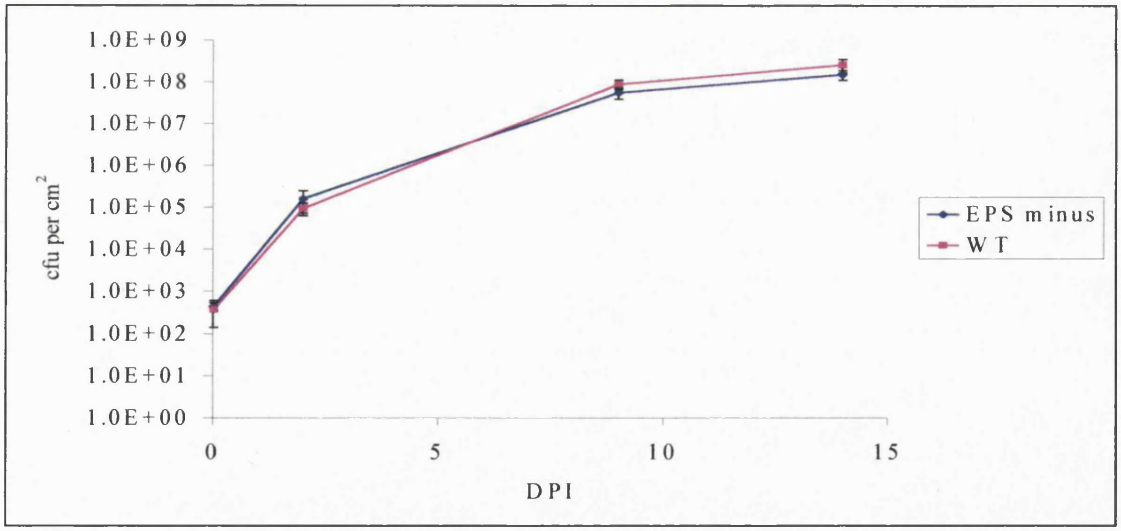


Figure 4.12 Growth of EPS⁻ and WT bacteria at initial infection point.
A suspension of $1x10^6$ bacteria per ml was infiltrated into cassava leaves using a needleless syringe. The area of infection was collected and the number of bacteria in each infection point calculated. Each point represents the mean of six individual plants, each determined by a counting the number of cfu in three replicate dilutions. A two-tailed t test revealed no significant difference between the number of cfu in WT or EPS⁻ infected leaves at any time point. CFU = colony forming units DPI = days post inoculation, error bars = ± standard error. Data obtained with the help of Alan Bryant.

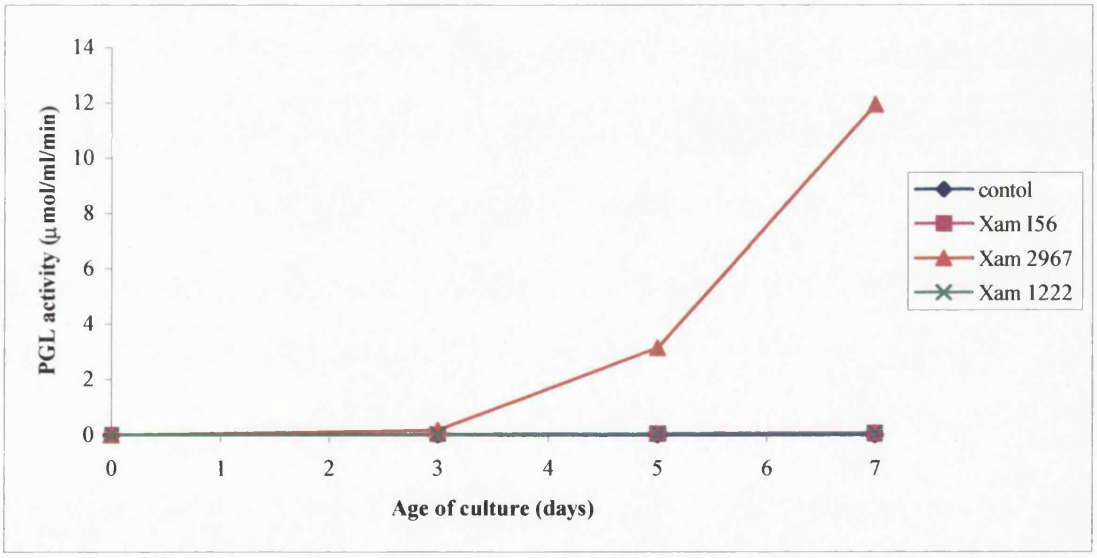


Figure 4.13 Pectate lyase production of *Xam*
Three strains of *Xam* were grown on minimal medium with cassava cell walls as the sole carbon source (Deshappriya, 1992). Strain 2967 produced significant quantities of pectate lyase *in vitro* of the three strains tested. Data points are the mean of two replicates, data obtained with the help of Kevin Geary.

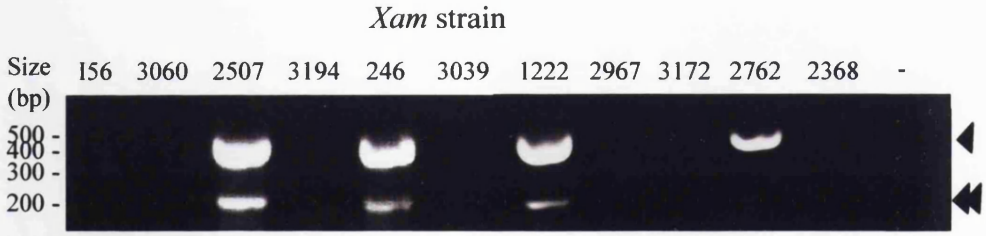


Figure 4.14 PCR screen for *pelA* in strains of *Xam*
Crude genomic DNA from 11 strains of *Xam* was subjected to PCR using primers designed against *pelA* from a number of phytopathogenic bacteria. Only four strains amplified products of the expected size (single arrowhead). Three of the positive strains also showed a lower band (double arrowhead) which was eliminated by increasing the annealing temperature. This indicates that *pelA* is not ubiquitous in the *Xam* population. However the data should be treated with caution as a *pelA* could be amplified from strain I56 when high quality genomic DNA was used as the template (see figure 4.16). Strains are indicated across the top of the gel, - = negative control with no template. Molecular weight is shown at the left hand side of the gel

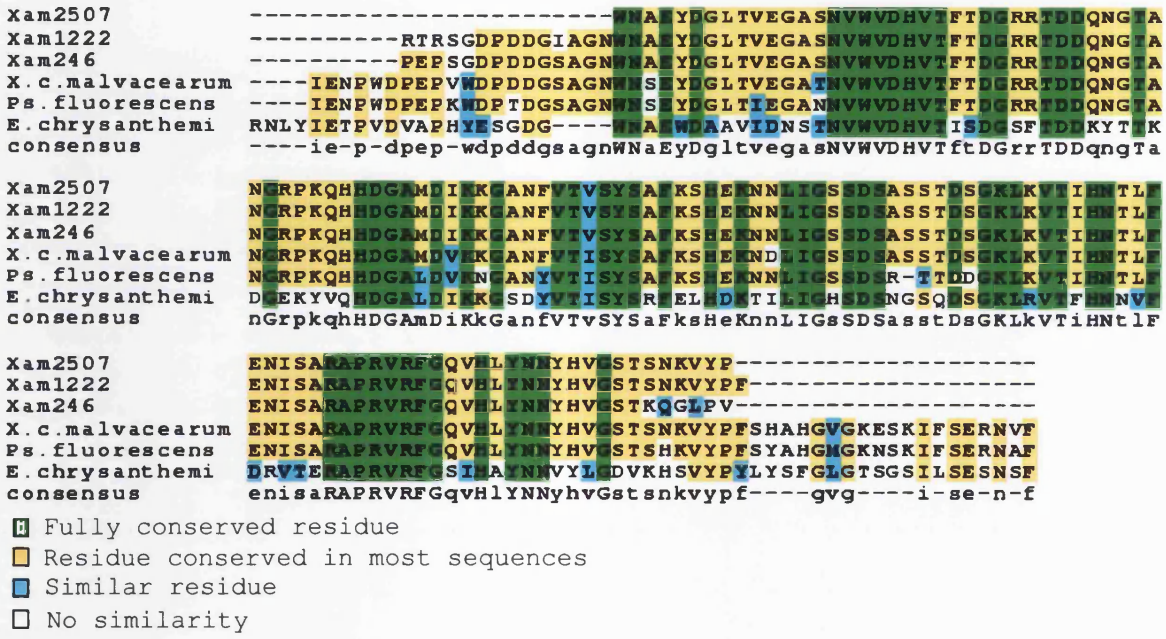


Figure 4.15 Alignment of partial pectate lyase protein sequences from four phytopathogenic bacteria.
Protein sequences from pectate lyase (Pel) enzymes of *Erwinia chrysanthemi* (E. chrysanthemi) (Keen and Tamaki, 1986), *Pseudomonas fluorescens* (Ps. fluorescens) (Liao, 1991) and *X. c.* pv. *malvacearum* (X. c. malvacearum) (Liao *et al.*, 1996) were aligned with the deduced protein sequence from three strains of *Xam* (Xam2507, Xam1222 and Xam246) (this study) using ClustalW (Thompson *et al.*, 1994). Fully conserved residues are shown in uppercase letters in the consensus sequence and partially conserved or similar residues are shown in lower case. The sequence from *E. chrysanthemi* is from the extracellular PelD, *P. fluorescens* and *X. c.* pv. *malvacearum* only produce one form of Pel (Liao *et al.*, 1996). The high level of sequence conservation between *Xam, P. fluorescens* and *X. c.* pv. *malvacearum* Pel enzymes can be seen. *E. chrysanthemi* PelD is more divergent, though the most similar to Xam Pel of any *pel* gene from *Erwinia* spp.

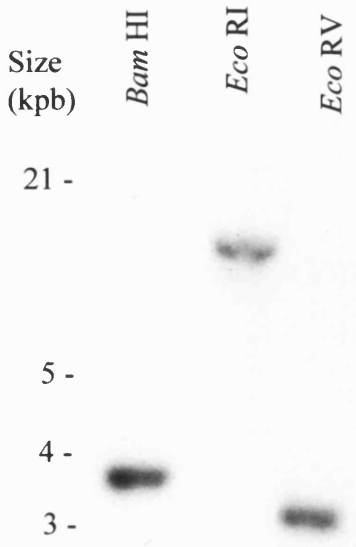


Figure 4.16 Southern blot of *Xam* I56 genomic DNA probed with 430bp of *pelA*. DNA was restricted with the enzymes indicated and fractionated on a 0.8% agarose TBE gel before transfer to Hybond N+ membrane. 430bp of *pelA* had previously been isolated from *Xam* using PCR primers designed against conserved regions in a number of *pelA* sequences. Size of molecular weight standards are indicated on the left hand side of the gel. This blot indicates that *pelA* is a single copy gene in *Xam*. The blot also shows that because of the large size of the *Eco* RI fragment containing *pelA* it is unlikely to be present in the genomic library constructed in this study. Molecular weights are indicated on the left hand side of the blot.

4.4 Discussion

This study is the first to report a definite pathogenicity or virulence factor for *X. axonopodis* pv. *manihotis* (*Xam*) infecting cassava. The pathogenicity of a highly virulent strain of *Xam* has been severely attenuated by the disruption of a single gene involved in the biosynthesis of extracellular polysaccharide (EPS). It is possible to argue that as EPS$^-$ *Xam* is unable to spread and cause many of the typical symptoms of CBB it is no longer pathogenic.

This study shows that the biosynthesis of xanthan in *Xam* is very similar to that of *X. c.* pv. *campestris*. The *gumD* genes from the two species are remarkably similar (89% identity and 93% similarity at the amino acid level) and that the gene organisation for *gumC-gumE* appears to be the same. *Xam gumD* is also very similar to the *gumD* homologue from *Xylella fastidiosa* (70% identical and 81% similar at the amino acid level). *X. oryzae* pv. *oryzae* also appears to have a similar organisation of *gum* genes to *X. c.* pv. *campestris* (Dharmapuri and Sonti, 1999) and it seems likely that the cluster is well conserved in xanthomonads. The lack of xanthan in *gumD* minus strains of *Xam* indicates that *gumD* has a similar role in *Xam* as in *X. c.* pv. *campestris*. However more detailed biochemical studies are needed to confirm this. It is possible that the deletion caused the whole *gum* operon to be inactivated but again more detailed studies are needed to confirm this.

EPS$^-$ *Xam* failed to induce any symptoms on cassava MCol22 when introduced *via* petiole stabs and produced much fewer symptoms than WT when introduced into the apoplast through leaf infiltration. Critically, virtually no spread of the *Xam* EPS$^-$ strain was detected away from the initial infection point. This appears to concur with other studies, where *X. oryzae* pv. *oryzae* mutated in *gumG* and showing no EPS were defective in spread from the initial infection point (Dharmapuri and Sonti, 1999) and *Ralstonia solanacearum* EPS$^-$ mutants multipled normally in stems near the site of wound inoculation but fail to spread and cause wilt symptoms on tomato (Denny, 1995). It is possible that a number of *X. c.* pv. *campestris* EPS$^-$ strains that have been described as "reduced virulence" cause less symptoms that WT bacteria because they are defective in spread. The *gum* gene cluster in *X. c.* pv. *campestris* appears to be expressed late during infection (Vojnov *et al.*, 2001) indicating that EPS is critical for later stages of infection.

In planta EPS$^-$ bacteria appeared to grow as well as WT bacteria in the immediate inoculation zone but caused significantly fewer symptoms on cassava plants. Similarly *X. oryzae* pv. *oryzae* mutated in the regulatory gene *rpfC* and showing severely reduced EPS grew at a similar rate to wild type but caused significantly less symptoms on rice plants (Tang *et al.*, 1996). Because of the method used to estimate bacterial numbers (the leaf surface was washed in sterile water but not surface sterilised) it is possible that some of the

numbers detected may have come from epiphytic population remaining on the surface post-inoculation. This could overemphasise the apparent growth of the EPS$^-$ strain as a "pathogen". However whilst *Xam* has been reported to grow as an epiphyte (Daniels and Boher, 1985) work by the author and others has failed to establish that *Xam* will grow epiphytically under glasshouse conditions (unpublished data; Blackwell, 2001). EPS is involved in epiphytic survival of *X. c.* pv. *campestris* (Poplawsky and Chun, 1998) and one might expect that numbers of epiphytic EPS$^-$ bacteria would be less than WT epiphytes. Whilst epiphytic populations may lead to an incorrect estimate of absolute number of pathogenic bacteria the comparison between EPS$^-$ and WT indicates that both are able to multiply in cassava.

The mechanics of the role EPS plays in bacterial pathogenesis have long been speculated upon (e.g. Leach *et al.,* 1957). Various functions have been proposed, including protection from dehydration, concentrating minerals and nutrients, reducing contact with toxic compounds, enhancing attachment to surfaces and enhancing spread through the plant (Denny, 1995). EPS may aid spread either by physical means or by modifying the intercellular environment in advance of the bacteria making colonisation more likely. Forcing bacteria throughout the apoplast by creating hydrostatic pressure may be a crucial role for EPS. EPS from *Erwinia amylovora* is highly hydrophilic and may cause "swelling pressure" enabling the bacterium to invade deeper into plant tissue (Schouten, 1990). Many xanthomonads are non-motile or only show motility under some conditions, so EPS may provide a mechanism of movement (Kamoun and Kado, 1990). *X. c.* pv. *campestris* can either be motile or produce EPS, but not both; the motile bacteria have reduced virulence until they switch to producing EPS when normal virulence returns (Kamouna and Kado, 1990). In contrast *X. oryzae* pv. *oryzae* is motile and produces EPS, but EPS$^-$ mutants that show reduced spread retain motility, indicating that motility does not contribute to spread in the plant but EPS does (Dharmapuri and Sonti, 1999).

EPS from *Xam* has been observed in cassava in advance of infecting bacteria (Boher *et al.,* 1995). Its function may be to modify the intercellular environment to facilitate colonisation. EPS is extremely hydrophilic and is thought to contribute to the water-soaking seen in infected leaves (Denny, 1995). In *Ps. syringae* infected plants up to one third of the wet weight of a water-soaked leaf can be attributed to hydrated EPS (Fett and Dunn, 1989). It is possible that when EPS$^-$ *Xam* were infiltrated into leaves, enough water was co-infiltrated for initial bacterial growth. However, once the infection site was saturated with bacteria the available water in other leaf areas presumably would have been too low to support bacterial growth. A similar case may be made for the concentration of minerals and nutrients by xanthan.

Another potential role for EPS that could impact on the ability of EPS⁻ *Xam* to spread is as a protectant against host defence responses. Local defence responses are probably suppressed by *hrp*-delivered effector proteins (Lahaye and Bonas, 2001). Indeed EPS minus strains of *X. c.* pv. *campestris* inhibit papillae formation to levels similar to that with WT strains (Bestwick *et al.*, 1995). However *hrp*-dependent defence suppression relies on close proximity of plant cell and pathogen (Grant and Mansfield, 1999) and it is possible that EPS⁻ *Xam* are susceptible to host responses produced at a distance from the initial infection site. Constitutive phenolics, some with potential antimicrobial effects, have been characterised from cassava (Gomez-Vasquez de Beeching, 2000); also phenolics were localised to *Xam* EPS in infected cassava xylem (Kpémoua *et al.*, 1996). The authors suggested that the binding of these compounds to the EPS may protect the bacteria from the antimicrobials. EPS from animal pathogenic bacteria has long been known to enhance resistance to antibiotics (Stewart and Costerton, 2001). EPS from a number of plant pathogenic bacteria is involved in protecting cells from reactive oxygen species. (ROS) (Király *et al.*, 1997). Cassava cells produce ROS in response to *Xam* both *in vitro* and in challenged leaves (Day, 2001 but the levels produced may not be sufficient to kill *Xam* (unpublished data; Stannard, 2001). However, it is impossible to say what concentrations of ROS are experienced locally by the pathogen.

In addition to infecting leaves, *Xam* also infects cassava xylem; artificial infection by petiole stabbing introduces bacteria into the xylem mimicking this phase of the disease. The failure to induce any symptoms in petioles infected with EPS⁻ *Xam* again indicates a critical role for EPS in *Xam* systemic invasion and pathogenicity. In addition to the roles detailed above, EPS may be involved in blocking the xylem and causing water stress symptoms in vascular diseases (Denny, 1995).

EPS probably has other roles that were not tested for in this study. *X. c.* pv. *campestris* mutated in the regulatory gene *pigB* did not produce EPS or xanthomonadin and showed reduced epiphytic survival (Poplawsky and Chun, 1998). Whilst xanthomonadin is known as a UV protectant EPS is speculated to be involved in resisting dehydration stresses (Poplawsky and Chun, 1998). *Xam* has to survive extreme conditions to live as an epiphyte (Daniels and Boher, 1985) and it will be interesting to see the effect EPS has on epiphytic survival.

Pectate lyase (pel) activity has previously been reported from *Xam* (Deshappriya, 1992). This work is confirmed here in finding pectate lyase activity in strain 2967 but not in other strains of *Xam*. Pel is a pathogenicity factor in a number of soft-rotting bacteria (Collmer and Keen, 1986), but a role for Pel in the pathogenicity of xanthomonads has yet to be clearly shown (Liao *et al.*, 1996). Not all xanthomonads produce Pel (Dye, 1960) and

initial PCR from a number of *Xam* strains appeared to confirm this. Of the 11 strains tested only four, not including strain I56, gave a product of the predicted size. Subsequent PCR using highly purified DNA from strain I56 gave an amplification product, indicating that the failure to amplify *pel* homologues from at least some of the strains was due to contaminants in the genomic DNA preps. PCR products were later sequenced and showed a high degree of similarity with *pel* genes from other xanthomonads. However both strain 1222 and strain I56 failed to show Pel activity *in vitro* although they contain DNA with high similarity to *pel*. Strain I56 appears to have one copy of a gene similar to *pel* in its genome, though further investigation is needed to confirm this. There is evidence that strains of *X. c.* pv. *vesicatoria* that do not produce Pel *in vitro* may contain a copy of the *pel* gene (Beaulieu *et al.*, 1991). It is possible that Pel is only produced in response to plant-derived signals in the majority of *Xam* strains and strains such as 2967 are unusual in producing Pel *in vitro.* Various pathovars of *Xanthomonas* mutated so they lost production of Pel appeared to retain full virulence (Beaulieu *et al.*, 1991, Dow *et al.*, 1989). It has been suggested that Pel is required by xanthomonads for an as yet uncharacterised saprophytic stage (Liao *et al.*, 1996). The role of Pel in the pathogenesis of *Xam* has still to be unravelled. However, because *pel* appears to be a single copy gene in *Xam* and gene disruption technology has been found to work in *Xam* during this study, creation of a *pel* minus strain of *Xam* should be practicable.

Chapter 5

Preformed Defence Mechanisms of Cassava

5.1 Introduction

The preformed component of plant defence is the reason for most plants being resistant to most pathogens (Dangl and Jones, 2001). Preformed defences are extensively involved in non-host resistance (Heath, 2000a) as well as being implicated in some cases of host resistance (Osbourn, 1996). This chapter investigates potential preformed components of the cassava defence armoury. The three areas under study are the potential of stomatal distribution as a component of resistance, antimicrobial proteins present in cassava latex and characterisation of antimicrobial compounds in cassava leaves.

Unlike fungal pathogens, some of which are able to enter the host actively (Knogge, 1996), bacterial pathogens rely on passive entry into the plant (Wood, 1967). The main entry points are stomata, other natural openings and wounds (Alfano and Collmer, 1996). A number of plant pathogenic bacteria enter the host through stomates including many *Pseudomonas syringae* pathovars and several *X. campestris* pathovars (Huang, 1984). Stomatal features may play a role in disease susceptibility in some systems (see introduction). The adaxial surface of cassava leaves has been investigated as a potential entry point for *Xam* (R. Day and R. Cooper personal communication). Because of large quantities of surface wax on the abaxial leaf surface it is completely non-wettable. This indicates that if bacteria enter cassava through the stomates they are likely to enter through the adaxial surface, which is wettable. When cassava leaves were sprayed with fluorescent particles the suspension dries along the main vein, where adaxial stomates are located. This indicates a potential concentration of bacteria spread by rain splash over the points of entry (R. Day and R. Cooper personal communication). Cassava was originally thought to have no adaxial stomates (El-Sharkawy *et al.,* 1984). However El-Sharkawy and co-workers carried out a detailed study and showed that cassava cultivars have significant numbers of functional adaxial stomates, though they are confined to the main veins (El-Sharkawy *et al.,* 1984). It was decided to investigate if the number and distribution of stomates on the adaxial surface of cassava leaves are a component of resistance to CBB.

Latex is the cytoplasm of highly specialised cells called laticifers, which form a complex network in a large number of plant species (Farrell *et al.*, 1991). When laticifers are ruptured latex exudes from the wound (Martin, 1991). There is no definite role assigned for latex, which is remarkable considering how widepsread a phenomenon latex production is (latex production has been reported for 20 plant families (Metcalfe, 1967)). However the function of latex *in planta* has been speculated to be in defence because of a number of

circumstantial observations, including the constitutive presence of antimicrobial compounds, proteins and transcripts from defence related genes. In order to investigate the presence of constitutive antimicrobial enzymes in cassava latex various known defence-related enzymes were assayed for and an EST library of cassava latex was constructed.

Plants contain a vast range of secondary metabolites and the role of some of these is either known or speculated to be in defence (Dixon, 2001). Antimicrobial secondary metabolites are grouped into two classes dependent on whether they are induced on pathogen attack (phytoalexins) or present constitutively (phytoanticipins). Phytoanticipins are produced by most plant families and have been shown to play a role in both non-host and host resistance. The accumulation of phytoalexins has been correlated with defence because it occurs in response to infection, at the site of infection and in sufficient quantities to be toxic to the invading pathogen (Hammerschmidt, 1999). A role for phytoalexins in defence against pathogens has also been inferred by the discovery of pathogen phytoalexin detoxifying enzymes that are essential for pathogenicity (VanEtten *et al.,* 2001). Antimicrobial compounds from plants are chemically diverse and include terpenoids (e.g. rishitin, a phytoalexin from tobacco), aliphatic acid derivatives (e.g. tuliposide A, a phytoanticipin from tulips), phenolics and phenylpropanoids (e.g. resveratrol, a phytoalexin from grape) and nitrogen and/or sulphur containing compounds (e.g. camalexin, a phytoalexin from *A. thaliana*) (Dixon, 2001). The discovery of novel antimicrobials usually involves the extraction of low molecular weight compounds from pathogen challenged and control plants with a variety of polar and non-polar solvents and testing the extracts by an antimicrobial assay. Antimicrobial extracts would then be separated using a variety of techniques (TLC, HPLC, GC) to try and identify (GC-MS, NMR) the compound(s) responsible for the antimicrobial activity. This approach was taken to discover phytoalexins produced by cocoa in response to *Verticillium dahliae* infection, a system where no phytoalexins were known (Cooper *et al.,* 1996). Four phytoalexins were discovered; the pentacyclic triterpene arjunolic acid, two hydroxylated acetiophenones and elemental sulphur (Cooper *et al.*, 1996). This was the first report of an inorganic antimicrobial produced by plants and elemental sulphur has now been found in a number of plants in response to vascular infection (Williams *et al.,* 2002). No attempt has been made to investigate putative antimicrobial compounds from cassava aerial tissues. Therefore a study to discover putative, novel antimicrobial compounds from cassava was conducted. The potential of cassava to produce both phytoalexins and phytoanticipins was investigated.

5.2 Materials and Methods

5.2.1 Stomatal imprints and estimation of density

The adaxial surfaces of fully opened leaves were sprayed with a clear plastic fixative spray (Sprayway, USA) and left to dry. The film of plastic was peeled off with a clear, 6cm wide, plastic adhesive tape (Sellotape, Dunstable, UK) to yield a permanent imprint of the leaf surface. Imprints were examined under at light microscope at 200x magnification. The diameter of the field of view at 200x magnification was *ca.* 1mm. The number of stomates per mm^2 was determined by first marking the leaf imprint along the midrib at the second, middle and penultimate 10% of leaf length then counting all visible stomates whilst scrolling between marks. The number of stomates counted was then divided by the area of the section investigated to give the number of stomates per mm^2. At least ten replicate leaf lobes were counted.

5.2.2 Extraction of cassava latex

Petioles were physically separated from the stems of cassava plants. The exuded latex was collected with a micropipettor, transferred to microfuge tubes and stored on ice. Approximately 10µl latex was exuded from each wound site.

5.2.3 Enzyme assays

5.2.3.1 Protease assay

General protease activity was determined by the release of red azo-dye labelled peptides from azocasein, adapted from the method of Plantner (1991). 20µl of latex was incubated in 100µl 2% azocasein (Sigma-Aldrich) in 50mM sodium phosphate buffer at pH 5.5, 6.0, 7.0 and 8.0 (see appendix 2). Samples were incubated at 37°C for 4 h and the reaction terminated with the addition of 400µl 20% trichloroacetic acid. Undigested azocasein was pelleted by centrifugation and free azo-peptides were assayed spectrophotometrically at 420nm.

5.2.3.2 Lysozyme assay

100µl latex was added to 600µl phosphate/NaCl/azide buffer (67mM phosphate, 15mM NaCl, 8mM azide, pH 6.3) and centrifuged at 10,000g for 15 min. 25µl of supernatant was added to 1ml assay buffer (0.2 mg ml^{-1} *Micrococcus lysodeikticus* cell walls (Sigma-Aldrich) in phosphate/NaCl/azide buffer) and the assay was conducted at 37°C. Activity was measured as a decrease in absorbance at 570nm.

5.2.3.3 Chitinase activity

Chitinase activity was assayed using the soluble dye labelled substrate, carboxymethyl-chitin-Remazol Brilliant Violet (Cm-chitin-RBV) (Loewe Biochimica GmbH, Germany). 100µl latex was added to 150µl substrate in a total volume of 850µl phosphate buffered saline (see appendix). The reaction was terminated with the addition of 150µl 1M HCl, undigested substrate was pelleted by centrifugation at 10,000g for 10 min and digested chitin detected spectrophotometrically at 595nm.

5.2.3.4 β-1,3 glucanase assay

Glucanase activity was detected as degradation of the labelled curdlan substrate Remazol Brilliant Blue (RBB)-curdlan (Loewe Biochimica GmbH) in an assay identical to the chitinase assay.

5.2.4 Extraction of latex RNA

Petioles were pulled from the stems of cassava plants. 1ml of the exuded latex (from approximately 100 petioles) was added directly to 10ml TRI reagent (Sigma-Aldrich) at room temperature. After each addition of latex the mixture was shaken to ensure inactivation of any RNases present in the latex. The mixture was then vortexed for 1 min before the addition of 3 ml chloroform and centrifugation at 10,000g for 10 min. RNA was precipitated from the aqueous layer with the addition of 0.7 volumes of isopropanol and centrifugation at 10,000g for 30 min. The RNA pellet was washed in 70% ethanol and dissolved in 500µl H_2O. RNA was quantified and checked for integrity on a 1% TBE gel.

5.2.5 Construction of cDNA library

The latex cDNA library was constructed as detailed in general materials and methods

5.2.6 Extraction of putative antimicrobial compounds

All glassware was washed in excess of the solvent used for extractions prior to use and extracts were protected from light to avoid damage to photolabile compounds.

Leaf tissue (1.5g) inoculated with *Pst* and mock-inoculated controls were ground to a fine powder in a pestle and mortar under liquid nitrogen. The powder was extracted in 15ml of a range of solvents from high to low polarity (methanol, ethyl acetate, diethyl ether and petroleum ether); all solvents were HPLC grade obtained from Merck Eurolabs. The extract was filtered through Whatman number 1 filter paper (Whatman International) to remove solids and further tested in antibacterial assays.

After initial experimentation the extraction process was refined as follows. Leaf tissue (20g) was ground to a powder as before and extracted for 3h in 50ml solvent at room temperature. The extract was centrifuged at 3000g for 15 min and the supernatant filtered through Whatman number 1 filter paper. The solvent was removed under reduced pressure and the dried extract was re-dissolved in 20ml of the same solvent in order to give a final equivalent of 1g fresh weight per ml.

5.2.7 Antibacterial assays in liquid culture

The extract to be tested was placed in polypropylene microfuge tubes. Polypropylene is resistant to damage by all solvents used in this study (Nunc Plasticwares catalogue, see www.nuncbrand.com). Solvents were removed under reduced pressure. 1 ml of a suspension of $1x10^6$cfu ml^{-1} *Xam* in NYGB was added to each tube and cultures grown overnight at 28°C shaking at 200 rpm. The optical density of the overnight culture was then determined at 600nm in a spectrophotometer. Solvent controls were included.

5.2.8 Thin Layer Chromatography (TLC) bioassays

Two solvent systems were used in this study: chloroform: ethyl acetate: methanol (2:2:1) (Stahl, 1969) and chloroform: ethyl acetate: formic acid (5:4:1) (Harborne, 1984). TLC tanks were washed in solvent mix and allowed to equilibrate for 1 hour. TLC plates (Silica gel 60 F254 on an aluminium backing, 20x20 cm, Merck Eurolabs) were run in solvent to remove any contaminants and allowed to dry. Extract was spotted onto TLC plates and resolved until the solvent front reached *ca.* 2 cm from the top of the plate. Solvent was allowed to evaporate for 2 h before the plate was transferred to a sterile plastic box in which humidity was kept high with water soaked tissues.

A weak agar overlay containing 0.1% glycerol, 0.8% nutrient broth, 0.5% agar and 0.05mg ml^{-1} TTC (2,3,5- triphenyl tetrazolium chloride) (Sigma-Aldrich) and seeded with *ca.* $1x10^6$ cfu ml^{-1} *Xam* was then applied. The plate with overlay was incubated overnight at 28°C. Red colouration indicated metabolically active bacteria.

5.3 Results

5.3.1 Stomatal distribution and density

Stomatal distribution and density over the adaxial surface was determined for susceptible variety MCol22 grown in the glasshouse. Leaf imprints from two leaf lobes were examined and the stomatal density recorded (figure 5.1). Stomates were concentrated along the midrib of leaves and towards the base with a maximal concentration of over 20 stomates per mm^2 found at the proximal end of the leaf. At the distal end of the leaf less than 5 stomates per mm^2 were observed. A few stomates were found near the main leaf veins but none were observed away from the veins (figure 5.1). The abaxial surface had a high density of stomates (several hundred per mm^2) and no variation was found over the surface.

For subsequent investigations it was decided to measure stomatal density (as the number of stomates per mm^2) for the second 10% of leaf length from the base within 1mm from the midrib on the adaxial surface. Although a higher stomatal density occurred in the basal 10% of leaves, difficulty was experienced obtaining leaf imprints from this area. Leaf imprints were taken from four glasshouse grown cassava cultivars and the stomatal density estimated microscopically. Stomatal densities from 10 leaves from each cultivar were determined; the mean stomatal distribution is shown on figure 5.2. Cultivar MCol22, which is the most susceptible of the four cultivars tested, showed significantly ($P<0.05$, t-test) fewer stomates than any other cultivar tested. The three field-resistant cultivars all showed stomatal densities of *ca.* 20 stomates per mm^2 with no significant difference between them. From these data it is unlikely that the relative resistance to CBB of MNga19, MNga1 and MVen77 compared to MCol22, in glasshouse conditions, is dependent on stomatal density.

With the purpose of determining if resistance to CBB in field grown cassava was related to stomatal distribution, stomatal imprints were taken from IITA test plots in Benin, West Africa. Leaves from two cassava cultivars, BEN (susceptible) and MNga2 (resistant, also known as TMS), were used to make imprints in Benin. The imprints were examined at the University of Bath. The stomatal distribution was determined at the leaf base (second 10% of leaf length from the base), middle (middle 10% of leaf length) and tip (second 10% of leaf length from the tip). Susceptible cultivar BEN had a significantly ($P<0.05$, t-test) higher stomatal density at the leaf base (33±1 stomates per mm^2) than resistant cultivar MNga2 (22±1 stomates per mm^2) (figure 5.3). BEN had a higher stomatal density at the leaf base than any of the cultivars previously tested (MCol22, MVen77, MNga1 and MNga19). The effect of growth conditions on stomatal density has not been elucidated so field grown and glasshouse grown cultivars should not be directly compared.

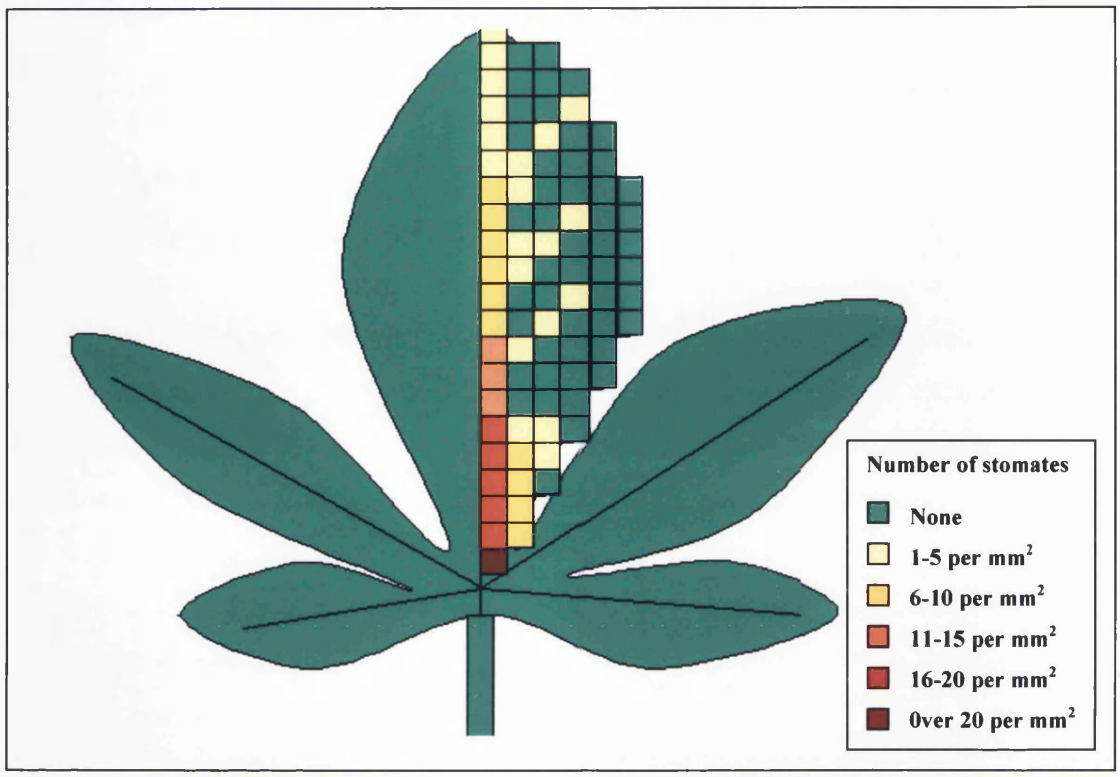


Figure 5.1 Adaxial stomatal density of cassava cultivar MCol22.
All the adaxial stomates from two lobes of glasshouse grown MCol22 were counted and their positions recorded. The stomatal density and distribution was estimated and mapped onto a cartoon of a cassava leaf. Similar adaxial stomatal distribution was found in other cultivars of cassava examined. Data obtained with the help of Elir Jones.

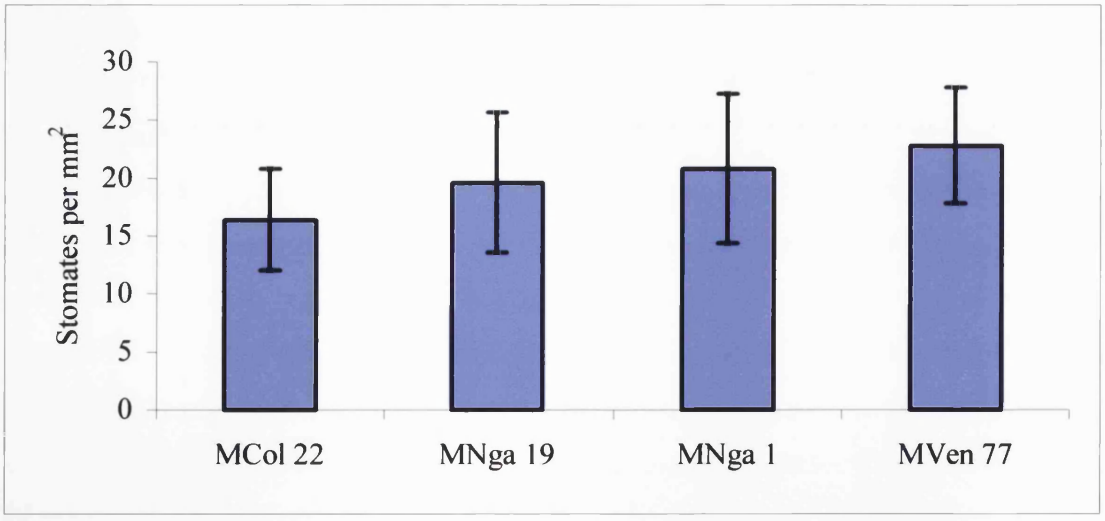


Figure 5.2 Adaxial stomatal density of glasshouse grown cassava plants.
The number of stomates per mm^2 was determined at the base of lobes from at least 10 separate leaves for each cultivar. MCol22 is regarded as susceptible to CBB whereas the other cultivars are deemed resistant. Error bars = ± standard deviation.

5.3.2 Antimicrobial enzymes from cassava latex

A number of known defence-related enzymes were assayed to see if they were present in cassava latex. Protease activity, manifest as an increase in non-precipitatable labelled peptides, was found at all pH values tested with the highest activity detected at pH 6 (figure 5.4). Lysozyme activity was tested against a suspension of *Micrococcus lysodeikticus* cell walls, after 2 h the OD 570 of a mixture of latex and cell walls had fallen from 0.673 to 0.189 (a drop of 70%) whereas a buffer control had fallen from 0.307 to 0.213 (a drop of 30%) (figure 5.5). However no control of just latex was included in this experiment and precipitation of latex may account for some of the decreased absorbance rather than solely the presence of lysozyme activity. The assay for chitinase activity in latex showed activity (by an increase in non-precipitable labelled oligosaccharides) but there was no increase for control tubes (figure 5.6). A similar assay showed the presence of β1-3 glucanase activity in cassava latex (figure 5.7).

5.3.3 EST library of cassava latex

Enzyme assays can only be used to detect known gene products and in order to determine potential antimicrobial proteins in cassava latex a small EST library from latex mRNA was constructed. RNA isolated from freshly harvested latex using Tri reagent with 1ml of latex yielded approximately 100μg of total RNA. From this total RNA 1.6μg of mRNA was isolated using the PolyATract system (Promega). The cDNA library was constructed according to manufacturer's instructions with all recommended controls to check the size and integrity of the cDNA (figure 5.8). After the primary library had been amplified, mass excision with the ExAssist helper phage was used to create a cDNA library in the plasmid vector pBluescript.

To check the integrity of the library, 10 random clones were grown up and sequenced in both directions (from the T3 and T7 priming sites in pBluescript). All 10 clones provided over 500bp of usable sequence in at least one direction. The cDNAs encoded for a variety of proteins including a chitinase and a β glucanase, both of which had previously been detected biochemically. On this basis 96 clones were sequenced to create the EST library. To ensure that all sequences were *bona fide* mRNAs the clones were sequenced with the T7 primer through the polyA tail of the cDNA. The failure rate of sequencing reactions in this direction is much higher than from the T3 primer (through the start codon) as Taq DNA polymerase sometimes "slips" when polymerising large sections of the same base (Paul Jones, personal communication). Over 75% of clones gave satisfactory sequence

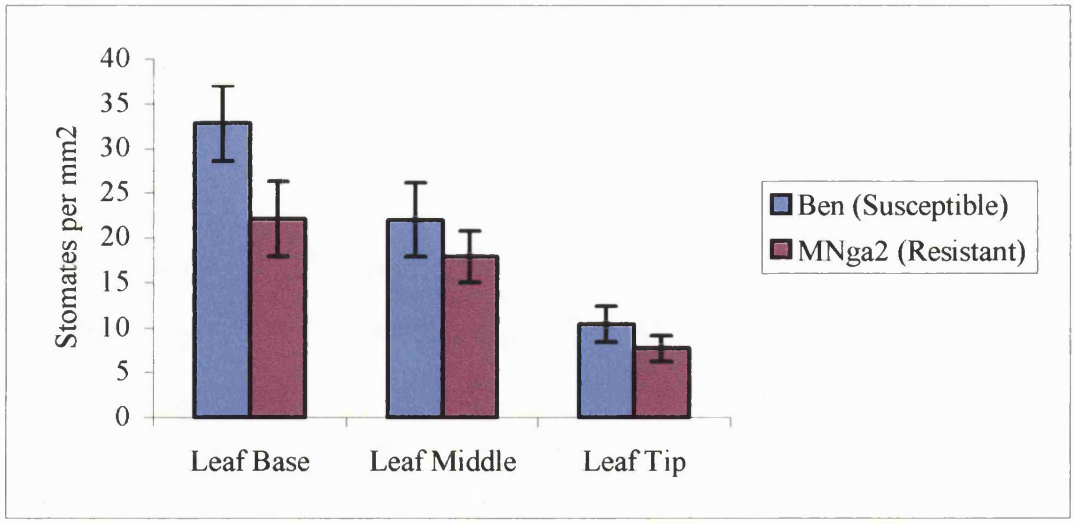


Figure 5.3 Adaxial stomatal density in two field-grown cassava cultivars.
Number of stomates per mm^2 was determined at three positions for two cassava cultivars; MNga2 (resistant to CBB) and Ben (susceptible to CBB). Estimates of density are the mean of at least 10 separate leaves. Error bars = ± standard deviation

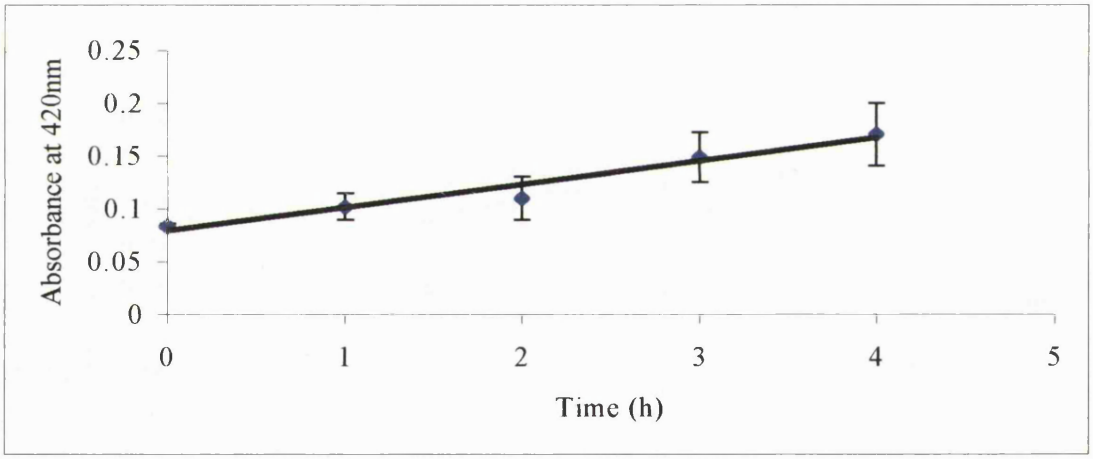


Figure 5.4 Protease activity of cassava latex.
Latex was incubated with azocasein in phosphate buffer at pH 6.0 and samples taken every hour. Activity was measured as an increase in absorbance at 420nm due to free azo-labeled peptides cleaved from azocasein over time. Protease activity was also observed at higher pH but was most marked at pH 6.0. Error bars = ± standard error. Data obtained with the help of Phil Minas

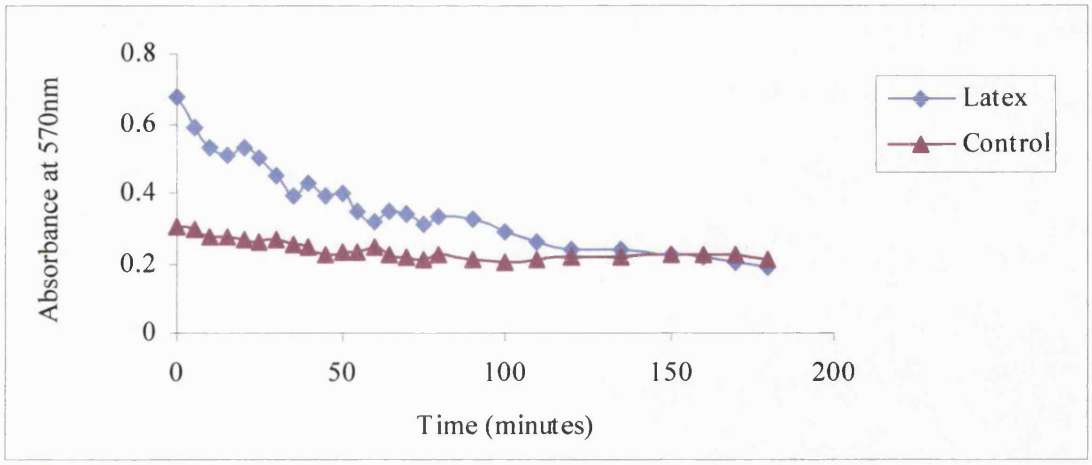


Figure 5.5 Lysozyme activity of cassava latex.
Micrococcus lysodeikticus cell walls in phosphate/NaCl/azide buffer were either incubated alone (control) or with cassava latex (latex). Whilst the optical denstity at 570nm of the control remained constant the optical density of cell walls incubated with latex decreased rapidly due to cleavage of the cell walls. The cell walls and latex showed a higher initial optical density than cell walls alone due to latex absorbing at 570nm. Data obtained with the help of Phil Minas

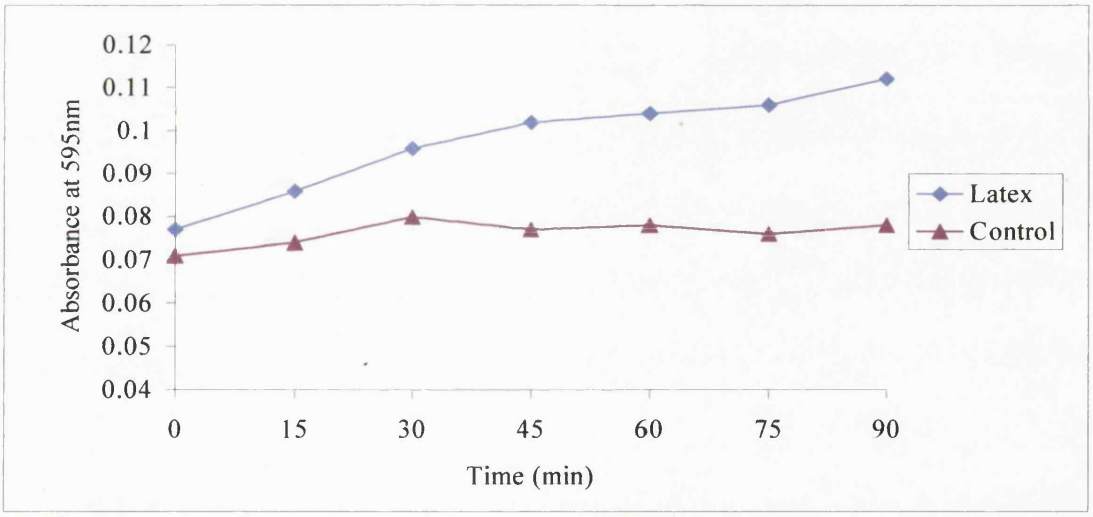


Figure 5.6 Chitinase activity of cassava latex.
Remazol Brilliant Violet labeled chitin in phosphate buffered saline was incubated alone (control) or with latex (latex). Release of oligosaccharides was detected as an increase in optical density at 595nm after acid precipitation of undigested polysaccharides. The control optical density remains constant whereas latex causes an increase in absorbancy indicating chitinase activity. Data obtained with the help of Phil Minas

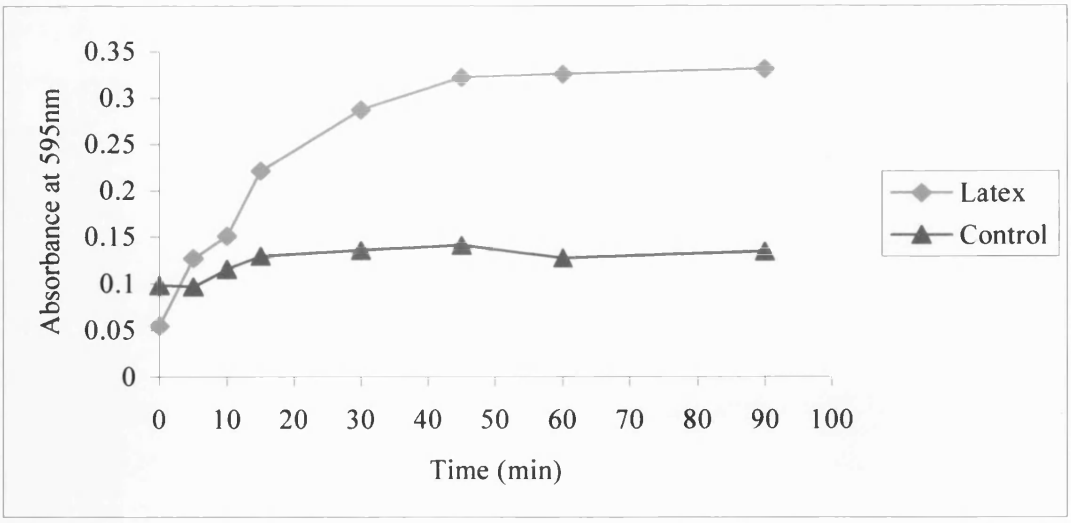


Figure 5.7 Endo-β-1,3 glucanase activity of cassava latex.
Remazol Brilliant Blue labeled curdlan was incubated in phosphate buffered saline either alone (control) or with latex (latex). Undigested curdlan was removed by acid precipitation and labeled oligosaccharides detected by measuring optical density at 595nm. The optical density of the control reaction remained constant whereas latex caused an increased release of labeled oligosaccharides indicating β -1,3 glucanase activity. Data obtained with the help of Phil Minas

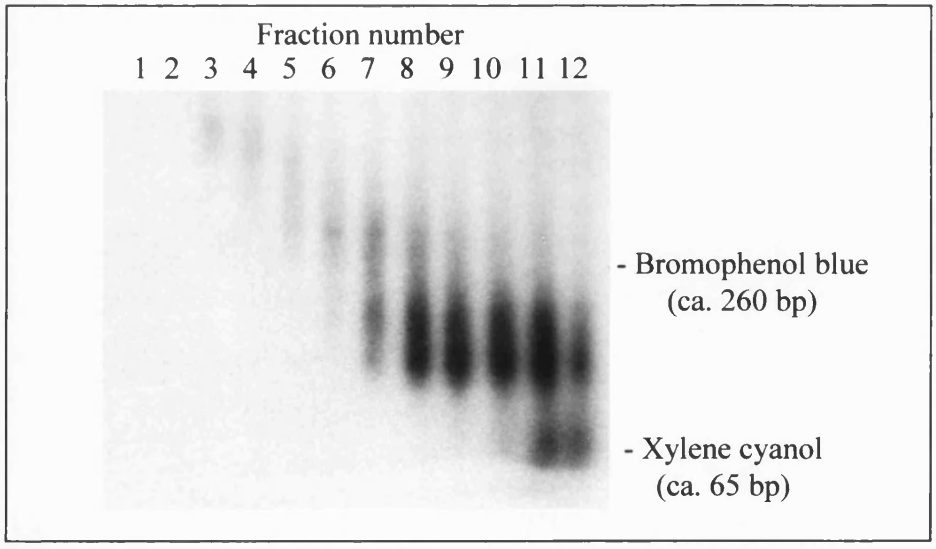


Figure 5.8 Size fractionation of latex cDNA. cDNA was synthesised from latex polyA RNA using the Zap-cDNA synthesis kit (Stratagene). Second strand cDNA was synthesised in the presence of [α^{32}P] dATP then fractionated on a Sepharose CL-2B drip column. 100µl fractions were collected from the column and 8µl run on a non-denaturing acrylamide gel. The gel was subjected to autoradiography in order to determine which fraction contained the longest cDNAs. Size was estimated by comparing with the migration of bromophenol blue and xylene cyanol in the loading buffer (Sambrook *et al.*, 1989) Fraction 3 was used to construct the cDNA library.

from the T7 primer and those that did not were sequenced with the T3 primer. Of the 106 clones sequenced 98% gave usable sequence data. The sequences were compared to sequences in the database using BlastX (Altschul *et al.*, 1997). The polyA tail was removed from sequences prior to analysis.

37% of ESTs appeared to encode proteins known to be involved in defence or stress responses; these included putative PR proteins (chitinases, protease inhibitors and glucanases), proteins involved in producing antimicrobials (linamarase, quinone oxidoreductase), proteins involved in protection against stress (drought induced proteins from *Arabidopsis* and methionine sulfoxide reductase, involved in repairing oxidative damage) and one EST with similarity to a protein involved in virus induced gene silencing (Argonaute). Of the rest 35% were from genes with a known function (including storage proteins, housekeeping proteins and enzymes involved in fatty acid biosynthesis) and 26% had no homology in the database or were similar to unknown genes from sequencing projects (figure 5.9 and appendix 6). Two clones did not contain an insert. Fifty eight different genes were sequenced (figure 5.9) showing that this library has a redundancy of 1 in 2. However the majority of this redundancy comes from two mRNA species; one encoding an acidic endochitinase and the other a storage protein. Indeed, 30% of the library is composed of clones encoding one of these two proteins. When the numbers of different genes are compared, 46% of genes are unknown, 29% are known but not involved in stress tolerance and 25% are either involved in tolerance to biotic or abiotic stress (figure 5.9).

5.3.4 Preformed antibacterial compound(s) from cassava leaves

The HR was induced on cassava leaves with *Pseudomonas syringae* pv. *tomato* (*Pst*) as a likely elicitor of phytoalexin production. Extracts from cassava leaves were assayed for antibacterial activity in 1ml liquid cultures of *Xam* with appropriate solvent controls. Ethyl acetate soluble extract from 0.1g hypersensitively responding tissue showed antibacterial activity in initial assays (figure 5.10). The antibacterial activity manifested itself as a reduction in *Xam* growth, rather than total inhibition. No other extract showed antibacterial activity.

In order to determine if 0.1g per ml was a physiologically relevent concentration the density of cassava tissue was estimated. 10g of cassava leaves were shown to displace approximately 10ml water in a measuring cylinder indicating that the initial concentration of extract used in antibacterial assays was lower than that *in planta.* For further experimentation an extract concentration of 1g leaves per ml of *Xam* culture was chosen as

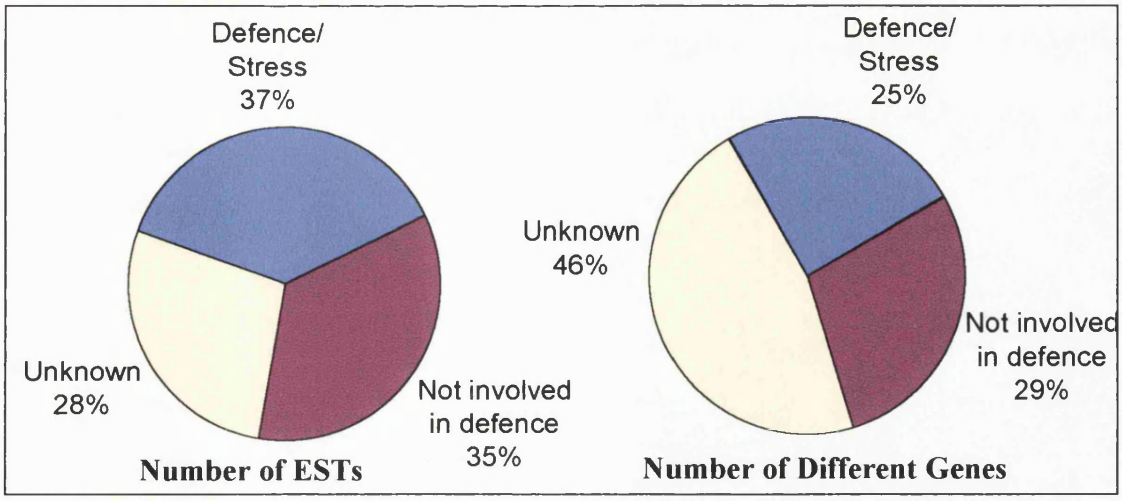


Figure 5.9 Hypothesized function of ESTs from cassava cDNA library
106 cassava ESTs were compared to the database using BlastX and grouped according to their possible function. 37% of total sequences (25% of the different genes found in the library) are speculated to be involved in defense against either biotic or abiotic stress. A full list of sequences is found in appendix 5.

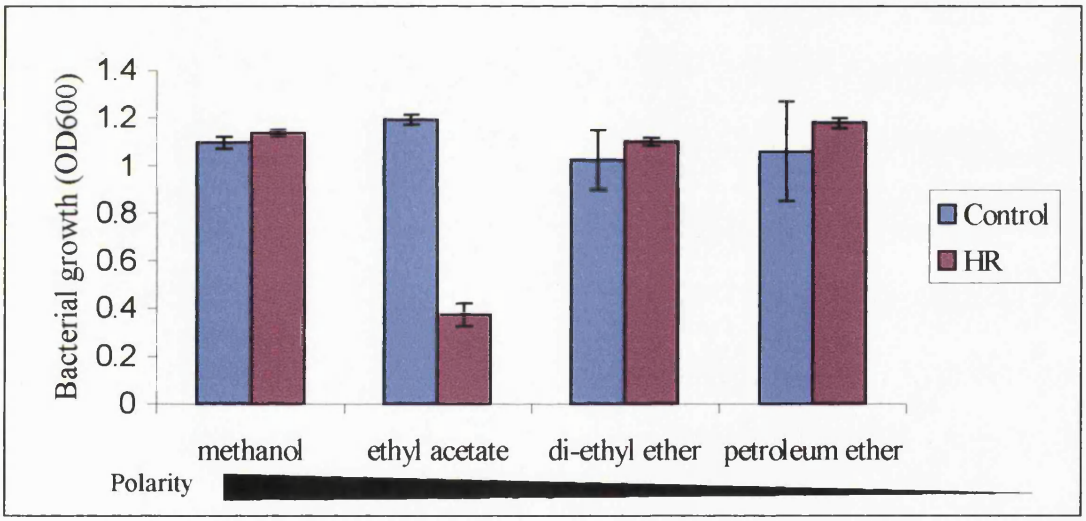


Figure 5.10 Growth of *Xam* strain I56 in extracts from hypersensitively responding MCol22 cassava leaves.
The HR was induced on cassava leaves by infiltration with *Pst* and left for 48 hours. Compounds were extracted from the leaves with a variety of solvents. Dried extract equivalent to that present in 0.1g fresh tissue was tested for antibacterial activity against *Xam* in 1ml cultures (HR). Solvent controls were performed to test for antibacterial contaminants in the solvent residues (Control). Bacterial growth was measured as OD at 600nm 36 hours after growth commenced. Only the ethyl acetate soluble extract had activity against *Xam*. Arrow shows decreasing polarity of solvents as determined by Reichardt (1990). Results are the mean of three replicates. Error bars = ± standard error

a concentration more likely to reflect the levels of antimicrobials in cassava leaves. Ethyl acetate and methanol soluble cassava extracts were tested for antibacterial activity at 1g ml^{-1}. Methanol soluble extracts showed a growth promoting activity, though extracts from hypersensitive tissue showed less growth promotion than from water inoculated tissue (figure 5.11). Ethyl acetate soluble extracts from both hypersensitivity responding and sham- inoculated leaves showed total inhibition of *Xam* growth (figure 5.11) indicating the presence of antibacterial compound(s) in this extract. Solvent controls showed equivalent growth to cultures with no extract present. Titrating the concentration of extract revealed that antibacterial activity was present in a dose dependent manner and resulted in total inhibition of bacterial growth at concentrations above 0.3g fresh weight per ml culture (figure 5.12). No major difference in the level of antibacterial activity was observed between challenged and unchallenged leaves.

In order to determine if *Xam* was more resistant to the antibacterial activity than other phytopathogenic bacteria *Pst* and *X. campestris* pv. *vesicatoria* were tested for susceptibility to the ethyl acteate soluble extract at a range of concentrations. Both were found to be susceptible over a similar concentration range to *Xam* (the data for *X. campestris* pv. *vesicatoria* is shown in figure 5.13). To see if the levels of antibacterial activity varied with cassava cultivar ethyl acetate soluble was tested from MCol22, MNga2, MNga19 and MVen77. All were found to have a similar level of antibacterial activity against *Xam ca.* 0.3g fresh weight per ml culture (figure 5.14).

The possibility that antibacterial activty was a result of reaction with ethyl acetate was tested by extracting cassava leaves with chloroform, which has a similar polarity to ethyl acetate (Reichardt, 1990). No growth inhibition occured in 1ml *Xam* liquid cultures incubated with chloroform soluble extracts from 2g fresh weight.

5.3.4.1 TLC bioassay development

In order to find a *Xam* strain suitable for the development of a TLC bioassay, NYGA plates supplemented with 1mg ml^{-1} 2,3,5- triphenyl tetrazolium chloride (TTC) were streaked with 8 different *Xam* strains from glycerol stocks. No growth was seen with any strain. In contrast *Pst* showed both good growth on TTC supplemented plates and the characteristic red colouration caused by the formazan product. When a large number (*ca.* $1x10^9$) of log phase *Xam* cells were added to 1ml agar supplemented with TTC only strain I56 produced red formazan after 24 h incubation, other strains showed no colouration even after a week long incubation.

To determine if the ethyl acetate soluble extract would inhibit *Xam* I56 on a TLC bioassay a series of dilutions of the extract were spotted onto a TLC plate (from 0.01, 0.03, 0.05 and

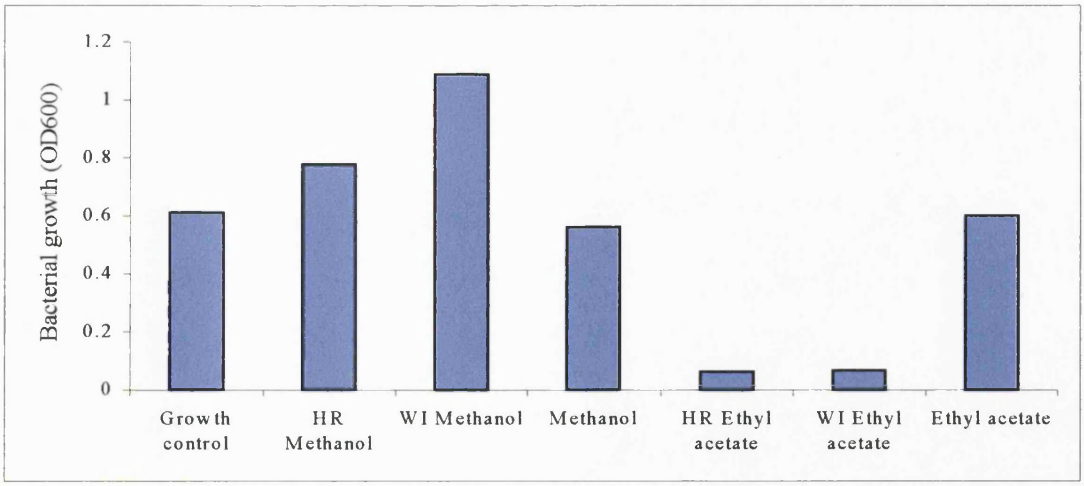


Fig. 5.11 Growth of *Xam* strain 2507 in methanol and ethyl acetate extracts from MCol22 cassava leaves.
Extracts from cassava leaves inoculated either with *Pst* (HR) or water (WI) were tested for their ability to prevent growth of *Xam* strain 2057. Dried extract from 1g fresh tissue was tested in 1ml cultures of *Xam*. Ethyl acetate soluble extract from both *Pst* and water inoculated leaves showed marked antibacterial properties. Methanol soluble extracts showed growth-promoting activities when compared to growth control (no solvent) and methanol solvent control (solvent evaporated to dryness). No inhibition of growth was seen is solvent controls when compared to the growth control.

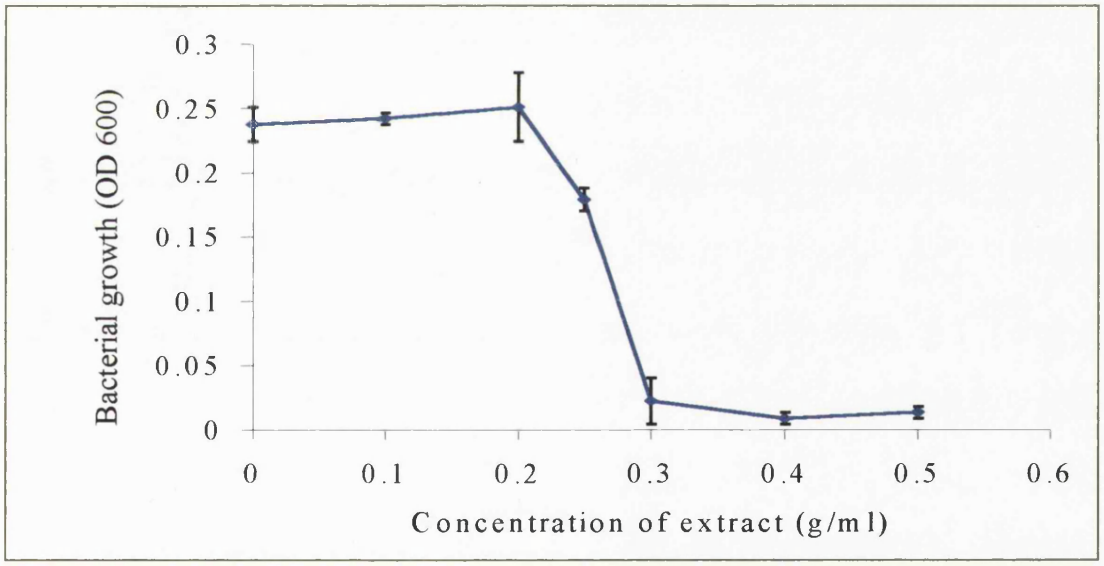


Figure 5.12 Concentration of ethyl acetate extract required to inhibit *Xam* growth.
Xam was incubated in 1ml cultures in the presence of various concentrations of ethyl acetate soluble extract from unchallenged cassava leaves. No growth was observed with the extract from 0.3g of cassava leaf and higher. Results are the mean of three samples. Error bars = ± standard error. Data obtained with the help of Chris Stannard

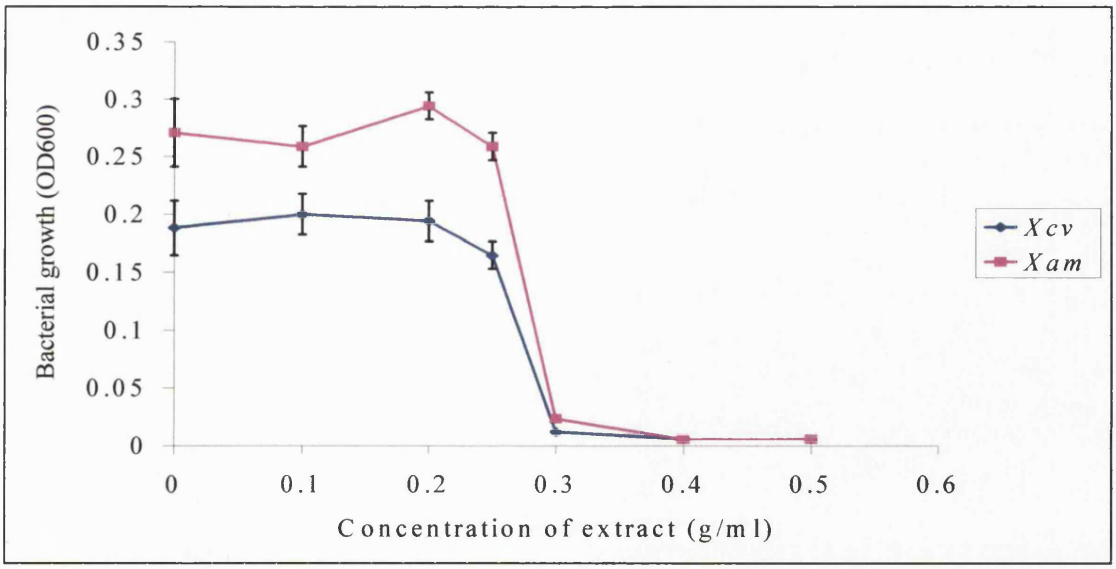


Figure 5.13 Inhibition of *Xam* I56 and *Xcv* by ethyl acetate extract
Xam and *Xcv* were incubated in 1ml cultures with increasing concentrations of ethyl acetate soluble extract from unchallenged cassava leaves. Both bacteria showed a similar tolerance to the extract. Results are the mean of three replicates. Error bars = ± standard error. Data obtained with the help of Chris Stannard.

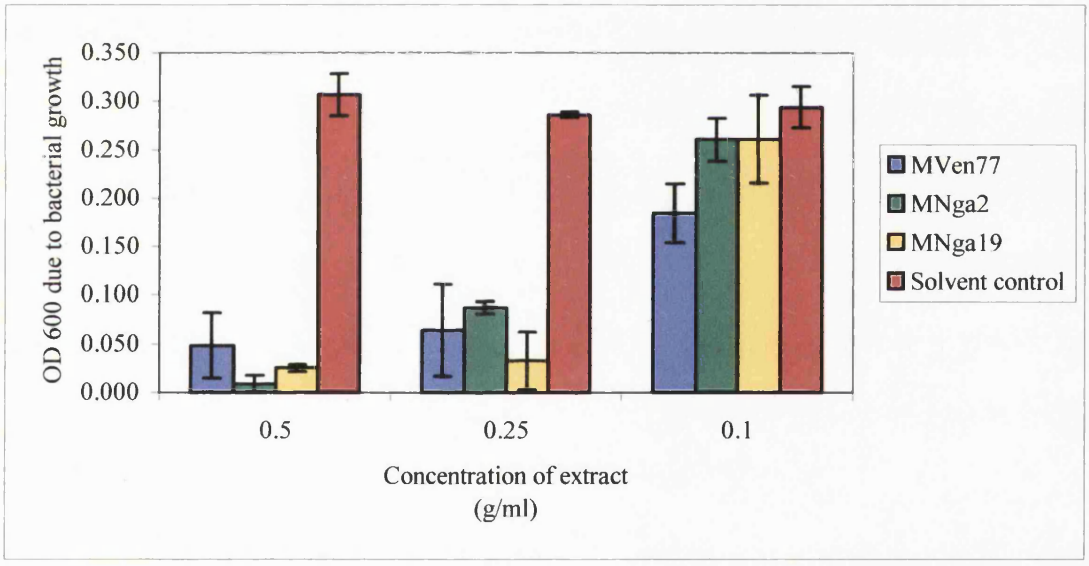


Figure 5.14 Level of antimicrobial activity in ethyl acetate soluble extract from three cassava cultivars
Xam strain 1222 was incubated with various concentrations of ethyl acetate soluble extract from cassava cultivars MVen77, MNga2 and MNga19. All extracts inhibited growth at 0.5 and 0.25 g/ml but did not inhibit growth at 0.1g/ml. This indicates that all cultivars of cassava tested have similar levels of antibacterial activity in the ethyl acetate soluble extract. Results are the average of two replicates. Error bars = ± standard deviation.

0.1g fresh tissue). An agar overlay containing $1x10^8$ cfu ml^{-1} *Xam* I56 and 1mg/ml TTC was applied to the dried TLC plate and left overnight. Growth inhibition (characterised as a clear spot with no red colouration) was observed around all extract concentrations from 0.03-0.1g fresh tissue (figure. 5.15). Ethyl acetate soluble extract from 0.05g fresh weight MCol22 leaves was subjected to TLC. Compounds that either quenched the fluorescence of a dye impregnated into the plates or fluoresced themselves under UV light were detected. With a solvent system of chloroform: ethyl acetate: methanol (2:2:1) seven bands were detected (table 5.1) and with a solvent system of chloroform: ethyl acetate: formic acid (5:4:1) nine bands were detected (table 5.2). An agar overlay applied to a TLC plate developed in chloroform: ethyl acetate: methanol showed growth inhibition but this inhibition could not be localised to a single band (figure 5.16). However this result proved to be unreproducible and all attempts to detect antibacterial activity with developed TLC plates either showed no red colouration or showed intense red colouration over the whole plate. In an assay for antifungal activity *Verticillium dahliae* fungal spores were applied to a TLC plate where ethyl acetate soluble extract had been developed in chloroform: ethyl acetate: formic acid (5:4:1). The plate was incubated for 1 week to allow fungal growth, after which hyphae were visualised with iodine. Whilst a zone of inhibition appeared to be present around *Rf* 0.75 the result was not conclusive.

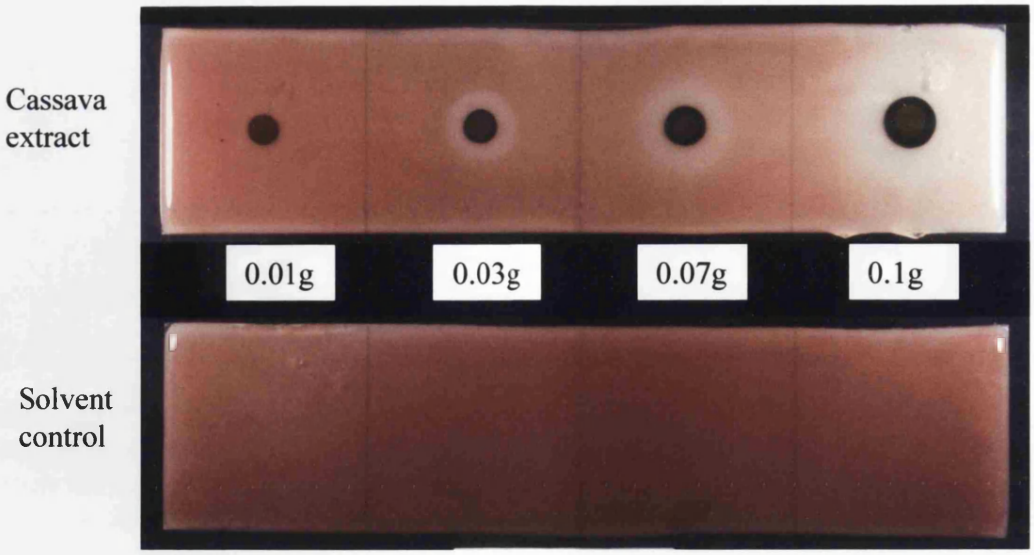


Figure 5.15 Preliminary TLC bioassay of ethyl acetate soluble extract from cassava leaves showing antibacterial activity.
Ethyl acetate soluble extract from cassava leaves was spotted on a Silica gel TLC plate, the weight of cassava leaves used to make the extract is indicated. The plate was allowed to dry and an agar overlay seeded with *Xam* and containing TTC applied. The plate was incubated overnight. Red colouration indicates bacterial metabolic activity, a halo of bacterial inhibition (no red colouration) can be seen around extracts from 0.03g cassava tissue and above. No inhibition is seen in the solvent control.

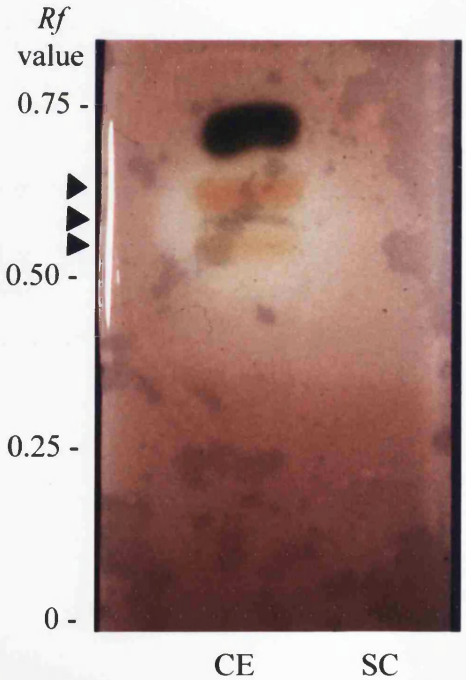


Figure 5.16 TLC bioassay of ethyl acetate soluble extract from cassava leaves showing antibacterial activity.
Ethyl acetate soluble extract from 0.1g of cassava leaves (CE) was separated on a Silica gel TLC plate with a solvent system of chloroform:ethyl acetate:methanol (2:2:1). The over lay was applied as detailed above. A halo of bacterial inhibition can be seen around bands between *Rf* 0.5 and 0.6 (indicated by black arrow heads). No inhibition is seen around the solvent control (SC). *Rf* values are calculated by dividing the distance migrated by the band by the distance migrated by the solvent front.

Rf value	Fluorescent at 360nm	Quenching at 255nm	Visible
0	✓	✓	
0.03		✓	
0.26		✓	
0.40		✓	✓
0.51		✓	✓
0.57	✓	✓	✓
0.71	✓	✓	✓

Table 5.1 *Rf* values and characteristics of bands from the ethyl acetate soluble extract from cassava leaves detected on TLC plates developed in chloroform:ethyl acetate:methanol (2:2:1).
Approximate *Rf* values were calculated by dividing the distance run by the band with the distance run by the solvent front. An example of the extract run in this solvent system (but with an agar overlay) can be seen in figure 5.16.

Rf Value	Fluorescent at 360nm	Quenching at 255nm	Visible
0		✓	
0.05		✓	
0.11		✓	
0.19	✓		
0.33		✓	
0.41		✓	✓
0.61		✓	✓
0.79	✓	✓	✓
0.85	✓	✓	✓

Table 5.2 *Rf* values and characteristics of bands from the ethyl acetate soluble extract from cassava leaves detected on TLC plates developed in chloroform: ethyl acetate: formic acid (5:4:1)
Approximate *Rf* values were calculated by dividing the distance run by the band with the distance run by the solvent front. Note that the visible bands run higher in this system than in the system in table 5.1.

5.4 Discussion

5.4.1 Stomatal density and distribution

It has been suggested that *Xam* enters cassava through stomates (Verdier *et al.*, 1990) and that adaxial stomatal distribution may play a role in resistance to CBB (R. Cooper personal communication). This study confirms the work of El-Sharkawy and co-workers (1984) showing that cassava cultivars have significant numbers of adaxial stomates, though they are confined to the main veins. However the density recorded in cultivar MCol22 (the only cultivar tested in both studies) is much lower (16 stomata per mm^2) than that of 67 stomata per mm^2 reported previously (El-Sharkawy *et al.*, 1984). Stomatal density is dependent on a number of environmental factors, including light levels; leaves of plants developing under high light conditions typically have a higher stomatal density than leaves of plants developing under low light intensity (Brownlee, 2001). Therefore it is unsurprising that the two estimates of stomatal density in MCol22 are not in agreement as conditions between the two studies (one in the field in Columbia and one in a glasshouse in Bath) are very different.

Of the four cultivars MCol22 is the most susceptible to CBB (see chapter 3, this thesis), but this study shows it had significantly fewer adaxial stomates than more resistant cultivars. For these varieties at least, stomatal distribution does not appear to contribute to their relative disease susceptibilities. However, field-grown plants of the susceptible cultivar BEN had more adaxial stomates than the resistant cultivar MNga2. This result is more in keeping with the original hypothesis and may indicate a role for stomatal distribution in resistance to CBB in the field.

Whilst this study found a gradient of adaxial stomatal density (low at the tip and high at the base of lobes) in both cultivar BEN and MNga2, recent work using plants grown in the same field as those in this study did not find this gradient (K. Wydra, personal communication). Stomatal density and distribution is dependent on the environmental conditions at the time of leaf development (Brownlee, 2001) and it is conceivable that a cassava leaf developing in the middle of the rainy season would have radically different stomatal density and distribution to a leaf developing during the dry season. For any conclusions to be drawn on the role of stomates in resistance to CBB, many more cultivars need to be examined, preferably under both field and controlled conditions, in conjunction with resistance testing. Stomatal density is a relatively easy phenotype to screen for and could be used as a marker for resistance in breeding programmes. Even though a significant difference was found between susceptible and resistant cultivars, enough stomates (at least 16 stomata per mm^2) to allow bacterial penetration were present on the

adaxial surface of all lines. Ideally a cultivar with no adaxial stomates should be identified and evaluated for disease resistance and could provide useful breeding material.

Stomatal characteristics may be involved in plant disease resistance to some pathogens although a definitive role has yet to be shown. Indeed many cases exist where variations in stomatal density, distribution, structure or function show no correlation with disease resistance (Royle, 1975). The role of stomatal density and distribution in disease resistance is difficult to prove. Whilst a survey of eight *Lycopersicon* species revealed a relationship between resistance to bacterial leaf spot caused by *X. campestris* pv. *vesicatoria* and frequency of stomata (Ramos *et al.,* 1992) the possibility remains that other factors were causing the differences in resistance. Recently a number of *A. thaliana* mutants with altered stomatal density, distribution and development have been described (van Groll and Altmann, 2001). If these were tested for resistance to bacterial diseases such as *X. c.* pv. *armoraciae,* which enters *A. thaliana* through the stomates (Hugouvieux *et al.,* 1998), perhaps some more robust evidence for the role of stomates in disease resistance could be obtained.

5.4.2 Antimicrobial enzymes from cassava latex

The identification of protease, lysozyme, chitinase and endo-β-1,3-glucanase activity in cassava latex confirms previous work on enzymes found in latex from other species. The latex from rubber tree (*H. brasiliensis*) (Kush *et al.,* 1990, Martin, 1991, Subroto *et al.,* 1996), opium poppy (Osmark *et al.,* 1998) and papaya (El Moussaoui *et al.,* 2001) all show constitutive PR protein activity.

The construction of a cassava latex EST library has revealed more about the possible functions of latex. Laticifer specific EST libraries have been constructed for *H. brasiliensis* (Han *et al.*, 2000) and poppy (Pilatzke-Wunderlich and Nessler, 2001). The largest subset of rubber tree latex ESTs were involved in rubber biosynthesis (16%), with 12.6% of the genes involved in defence and 27% similar to genes with no known function (Han *et al.*, 2000). The poppy latex EST library was used to study cell wall-degrading enzymes. Despite sequencing 4,500 clones only those involved in cell wall degradation were reported (Pilatzke-Wunderlich and Nessler, 2001).

The most abundant transcripts in the cassava latex EST library appeared to encode acidic endochitinases. Chitinases are classified as pathogenicity-related (PR) proteins and are grouped into three PR families, PR-3, -4 and –8 (van Loon and van Strien, 1999). Chitinase and chitinase/lysozyme activities have been detected in the latex of *H. brasiliensis* (Martin, 1991), fig (Glazer *et al.*, 1969), papaya (Howard and Glazer, 1967) and milkweed (Lynn, 1989). The high number of chitinase ESTs is not surprising

considering that chitinase and chitinase/lysozymes can represent up to 30% of total soluble protein in latex (Martin and Gaynor, 1988) and in *H. brasiliensis* an acidic chitinase represents over 10% of the total soluble protein (Martin, 1991). It is therefore surprising that no chitinase EST has been detected in *H. brasiliensis* latex EST library (Han *et al.*, 2000). The chitinase ESTs from cassava latex showed highest homology to a poplar chitinase rather than the more closely related *H. brasiliensis*. No substrate for chitinase activity has been detected *in planta* (Martin, 1991) and a role in defence against pathogens seems likely. Several chitinases have been shown to have antifungal properties (Kombrink and Somssich, 1997). The antifungal properties of chitinases result from weakening of the chitin components of the fungal cell wall. Fungi are under significant turgor pressure and a weakened cell wall results in cell lysis and death (Selitrennikoff, 2001). It is noteworthy that the majority of chitinase homologues were acidic. In tobacco, basic PR proteins tend to be expressed in response to wounding and acidic PR proteins in response to pathogen attack (Bol *et al.*, 1996).

Three ESTs showed highest homology to a β 1-3 glucanase from *H. brasiliensis*. β 1-3 glucanases are associated with plant defence (van Loon and van Strien, 1999) however they also have functional diversity. Unlike chitinase/ lysozymes, β 1-3 glucanases have substrates *in planta* including callose and the storage polysaccharide laminarin. β 1-3 glucanases have roles in developmental processes such as release of microspores into anther locules (Hird *et al.*, 1993), initiation of flower development (Neale *et al.*, 1990) and somatic embryogenesis (Helleboid *et al.*, 1998). β 1-3 glucanase activities have been reported in papaya latex (Clark and Stone, 1962) and poppy latex (Pilatzke-Wunderlich and Nessler, 2001). β 1-3 glucanases genes are expressed in the latex of *H. brasiliensis* (Chye and Cheung, 1995) and poppy (Pilatzke-Wunderlich and Nessler, 2001). In *H. brasiliensis* the levels of expression are much higher in latex than in other tissues (Chye and Cheung, 1995) whilst in poppy, expression was detected at similar levels in all tissues investigated (Pilatzke-Wunderlich and Nessler, 2001). This study clearly shows both gene expression and enzyme activities of β 1-3 glucanase in cassava latex. A recent report stated that β 1-3 glucanases found in latex were more likely to be involved in laticifer maturation than have a role in defence and that no role could be assigned for a β 1-3 glucanase homologue found in poppy (Pilatzke-Wunderlich and Nessler, 2001). When latex is extracted from rubber trees they are often over 20 years old (Kush *et al.*, 1990) and it seems unlikely that the lacticifers are not mature at this stage. Some β 1-3 glucanases have antifungal activity (Kombrink and Somssich, 1997) and they are expressed in response to pathogen attack (Linthorst, 1991). The antifungal properties of β 1-3 glucanases are

analogous to that of chitinases where a weakened cell wall results in cell lysis (Selitrennikoff, 2001). The bulk of evidence, albeit circumstantial, suggests a role in defence for β 1-3 glucanases. Chitinases and β 1-3 glucanases often act together to inhibit fungal growth *in vitro* and *in planta* (Jach *et al.,* 1995). For cassava, both ESTs and enzyme assays indicated the simultaneous presence of chitinases and β 1-3 glucanases.

Two ESTs appeared to encode trypsin protease inhibitors. Protease inhibitors are classed as PR proteins and are speculated to be involved in defence against herbivores, fungi, viruses and nematodes (Koiwa *et al.*, 1997; Ryan, 1990). Protease inhibitors also inhibit α-amylase activity from insects but not from mammals or bacteria (Schimoler-O'Rourke *et al.*, 2001). Protease inhibitors are known to have antifungal properties, though the mechanism of action is unclear (Selitrennikoff, 2001). Whilst high levels of protease inhibitor in the diet of insects will reduce growth, it is more likely that the inhibitors act as antifeedants (Ryan, 1990). The latex of papaya contains trypsin inhibitors and they are speculated to perform a defence function (El Moussaoui *et al.*, 2001) and genes encoding trypsin inhibitors are expressed in *H. brasiliensis* (Han *et al.*, 2000).

ESTs similar to two genes involved in the *A. thaliana* response to dehydration were found. Four ESTs were similar to RD22 and one EST was similar to an uncharacterised desiccation response protein from *A. thaliana.* RD22 mRNA is induced in response to water stress, salt stress and application of abscisic acid but is not induced in response to temperature changes (Yamaguchushinozaki and Shinozaki, 1993). Water stress induced ESTs were also detected in *H. brasiliensis* latex (Han *et al.*, 2000)

Two ESTs have similarity to an early nodulin from alfalfa. Nodulins are involved in the nodulation reactions of legumes in response to nitrogen-fixing bacteria (Long, 2001) but homologues of early nodulins have been found in a variety of non-nodulating plants.

Linamarase expression in cassava latex is well documented (Hughes, 1993; Elias *et al.,* 1997) and it is unsurprising that an EST representing linamarase should be isolated. Linamarase hydrolyses the cyanogenic glycoside linamarin to yield glucose and acetone cyanohydrin. Tissue damage resulting from herbivory would result in the exposure of linamarin to linamarase thus exposing the attacker to cyanide.

One EST appeared to encode a Hev b3-like protein. Hev b3 from rubber tree is similar to patatin and a number of stress-induced proteins from other species. Interestingly it is also an allergen, as are a number of latex proteins with simlarity to defence related proteins. PR proteins from groups 2, 3, 4, 5, 8, 10 and 14 show allergenicity in a number of species (Midoro-Horiuti *et al.*, 2001).

An EST with similarity to oxalate oxidase was detected. Oxalate oxidase is a member of the germin-like group of plant proteins, which have many roles in plant development and

defence (Bernier and Berna, 2001). It has been suggested that oxalate oxidase is a source of reactive oxygen species released on pathogen attack (Lamb and Dixon, 1997).

An EST with high similarity to the *A. thaliana argonaute* (*Ago1*) gene was detected in cassava latex. AGO1 is involved in translation initiation and is crucial to post-translational gene silencing (Fagard *et al.*, 2000). AGO1 also has homologues in fungi and animals. Interestingly, plant defence against viruses is often mediated by gene silencing (Waterhouse *et al.*, 2001). As insects often transmit viruses, latex must be exposed to viruses. It is likely that some form of defence against viruses is a component of laticifers.

Other ESTs were also found that appeared to be cassava homologues of genes involved in either defence against pathogens or stress responses. Methionine sulfoxide reductase counters the effect of oxidative damage to methonine residues in polypeptides (Sadanandom *et al.*, 2000). Ferritin sequesters iron thus limiting iron availability to invading pathogens (Briat *et al.*, 1999). Quinone oxidoreductases reduce quinones to hydroquinones or semiquinone radicals (Matvienko *et al.*, 2001). Quinones are secondary metabolites with roles in energy production, electron transport and host defence (Thomson, 1987). They are highly toxic both because of direct binding to biomolecules and by the generation of reactive oxygen species (O'Brien, 1991).

Latex is the cytoplasm of highly specialised cells and as expected some ESTs were similar to housekeeping genes. RNA helicase and a ribosomal protein were found in cassava latex, and have previously been detected in the latex of *H. brasiliensis* (Han *et al.*, 2000). Fifteen sequences were similar to vegetative storage proteins. It has been suggested that latex may also be a storage system (Maksymowch and Ledbetter, 1987) though cassava has a well-documented storage system in the tuber-like roots. The presence of storage protein transcripts in latex does not tally with latex having a role in defence; one would expect that the availability of nutrients to an invading pathogen would be limited in a defensive system. However production of vegetative storage proteins is increased in response to jasmonates, which have a variety of functions including modulating disease resistance pathways (Creelman and Mullet, 1997).

In contrast, the detection of ESTs with similarity to the major latex protein (MLP) is unsurprising. MLPs are laticifer-specific, low molecular weight proteins found in latex producing plants (Nessler, 1994). No definite role for MLPs has been assigned.

Several ESTs have similarity to genes involved in fatty acid biosynthesis. Two ESTs are similar to desaturases and one is similar to digalactosyldiacylglycerol synthase (DGDS). DGDS is involved in the biosynthesis of the galactolipid digalactosyldiacylglycerol, one of the major membrane lipids in plastids and thylakoids (Jorasch and Heinz, 1999). The localisation of DGDS is almost exclusively in the chloroplast envelope membrane

(Marechal *et al.*, 1997), structures that do not exist in latex. The homology of this EST to *A. thaliana* DGDS is high (51% identity and 71% similarity over 213 residues) but does not exclude the possibility that the EST encodes a gene similar to DGDS but with a different function. Latex from *H. brasiliensis* contains specialised vacuoles known as lutoids (Moir, 1959). It is possible that the DGDS homologue is involved in biosynthesis of lipids for the membrane surrounding lutoids.

Two ESTs have similarity to genes involved in plant cell wall degradation. The pectin methylesterase and xylan endohydrolase transcripts found in cassava latex may be involved in laticifer development. Pectin, cellulose and xylan-degrading enzymes have been detected in the latex from poppy and are speculated to have such a role (Pilatzke-Wunderlich and Nessler, 2001).

A role for cassava latex in defence against bacteria, fungi, insects and viruses is indicated by the sequences represented in the EST library. Some of the other proteins encoded by latex mRNA are probably involved in development of the lacticifer network, primary metabolism and the synthesis of secondary metabolites. Some of the genes with no known function to date may reveal biological function of latex.

5.4.3 Preformed antibacterial activity

Antimicrobial activity was detected in ethyl acetate soluble extract in cassava leaves. The antimicrobial extract killed or inhibited three phytopathogenic bacterial species, including *Xam*, at concentrations one third lower than that expected *in planta*. The levels of antibacterial activity appeared to be similar in all cultivars of cassava investigated and did not increase in response to incompatible pathogens.

The preformed nature of the antibacterial activity begs the question of what role it plays *in planta*. The strains of *Xam* susceptible to the extract are fully pathogenic on cassava. It is possible that the antibacterial compound(s) are located intracellularly so do not come into contact with *Xam* during the initial stages of infection. The first stages of *Xam* infection involve multiplication in the intercellular spaces and tissue damage does not occur until later on. If the antibacterial compound(s) were only released on cell death then *Xam* would not experience them until it had spread from the sight of initial infection. In contrast *Pst* infection induces rapid cell death so the antibacterial compounds would be released when bacterial numbers are low, thus halting the infection.

Xam may produce enzymes that detoxify the antibacterial compound(s) only *in planta*. Many genes involved in bacterial pathogenicity, such as some *hrp* genes, are only expressed *in planta* (Lahaye and Bonas, 2001). The phytoalexin-detoxifying enzyme pisatin demethylase from the fungal pathogen *Nectria haematococca* is glucose repressed

(VanEtten *et al* 1989) and any enzyme from *Xam* that detoxifies the antimicrobial compound(s) from cassava may be similarly regulated.

The lack of detectable antibacterial activity in chloroform extracts of cassava leaves points to another possible explanation. Whilst chloroform and ethyl acetate have very similar polarities (Reichardt, 1990) they have very different chemical structures. It is possible that ethyl acetate is reacting with a compound in cassava leaves and producing a toxic product.

In order to answer some of the above questions further characterisation of the antimicrobial extract was attempted. The development of a TLC bioassay system could have led to initial isolation and characterisation of the compound(s) responsible for the antimicrobial activity. However despite numerous efforts *Xam* proved to be unsuitable. The bioassay system of choice utilises an agar overlay containing the bacteria of interest and a tetrazolium growth indicator (Hamburger and Cordell, 1987). Metabolically active bacterial convert tetrazolium salts into the corresponding intensely coloured formazan *via* dehydrogenase activity (Hamburger and Cordell, 1987). The most commonly used tetrazolium salt for the detection of bacterial growth, 2,3,5- triphenyl tetrazolium chloride (TTC) appeared to inhibit growth of most strains of *Xam*. Although other tetrazolium salts are available they tend to be more toxic to bacteria (Hamburger and Cordell, 1987)

Despite a substantial collaborative effort (Stannard, 2001) a reproducible TLC bioassay using *Xam* was not developed. Therefore further characterisation of the antibacterial extract remains to be performed.

Chapter 6

Induced defence mechanisms of cassava

6.1 Introduction

Recent studies have shown that approximately 1% of the plant transcriptome is up-regulated in response to pathogen attack (Durrant *et al.*, 2000). With advances in differential display technologies it is becoming possible to isolate and characterise these up-regulated genes. Several techniques have been devised to detect differentially expressed mRNAs. These are either based on hybridisation of transcripts to DNA immobilised on a solid support (Nylon filter arrays, Microarrays and Oligonucleotide arrays) or analysis by gel electrophoresis (Differential display, Arbitrary primed PCR and cDNA-AFLP) (Baldwin *et al.*, 1999).

Solid-support arrays have the advantage of providing some quantification of gene expression levels. However at present they are expensive and only practicable when a large EST database is available for the organism under study (Baldwin *et al.*, 1999). Cassava does not at present have a large EST database (on 19th November 2001 there were 274 cassava nucleotide sequences in Genbank) and a gel-based assay is a more viable option.

cDNA-AFLP was developed to overcome some of the major problems associated with other gel-based differential display methods. In differential display, cDNA is subjected to PCR amplification using a poly-dT primer (3') and arbitrary primers (5') (Liang and Pardee, 1992). Arbitrary primed PCR uses arbitrary primers at both the 3' and 5' sites (Welsh *et al.*, 1992). However both these techniques require very low annealing temperatures that often result in non-specific amplification products and the suppression of rare transcripts (Bachem *et al.*, 1996).

In contrast, AFLP, originally developed for the fingerprinting of complex genomes, uses highly stringent PCR conditions (Vos *et al.*, 1995). DNA is digested with restriction enzymes and double stranded adapters are ligated to the restriction fragments. These adapters then serve as priming sites for PCR amplification. Selective bases are added onto the ends of primers to reduce the number of visualised bands (Vos *et al.*, 1995). cDNA-AFLP simply uses cDNA in place of genomic DNA as the template (Bachem *et al.*, 1996 and figure 6.1).

An investigation into the genes induced in tobacco suspension cells in response to the gene for gene interaction between *Cf-9* and *Avr9* was conducted by cDNA-AFLP (Durrant *et al.*, 2000). Early changes in response to *Avr9* were investigated and 290 differentially expressed genes were detected 15-30 min after challenge. These genes encoded a number of known signalling and defence related proteins along with some proteins of no known

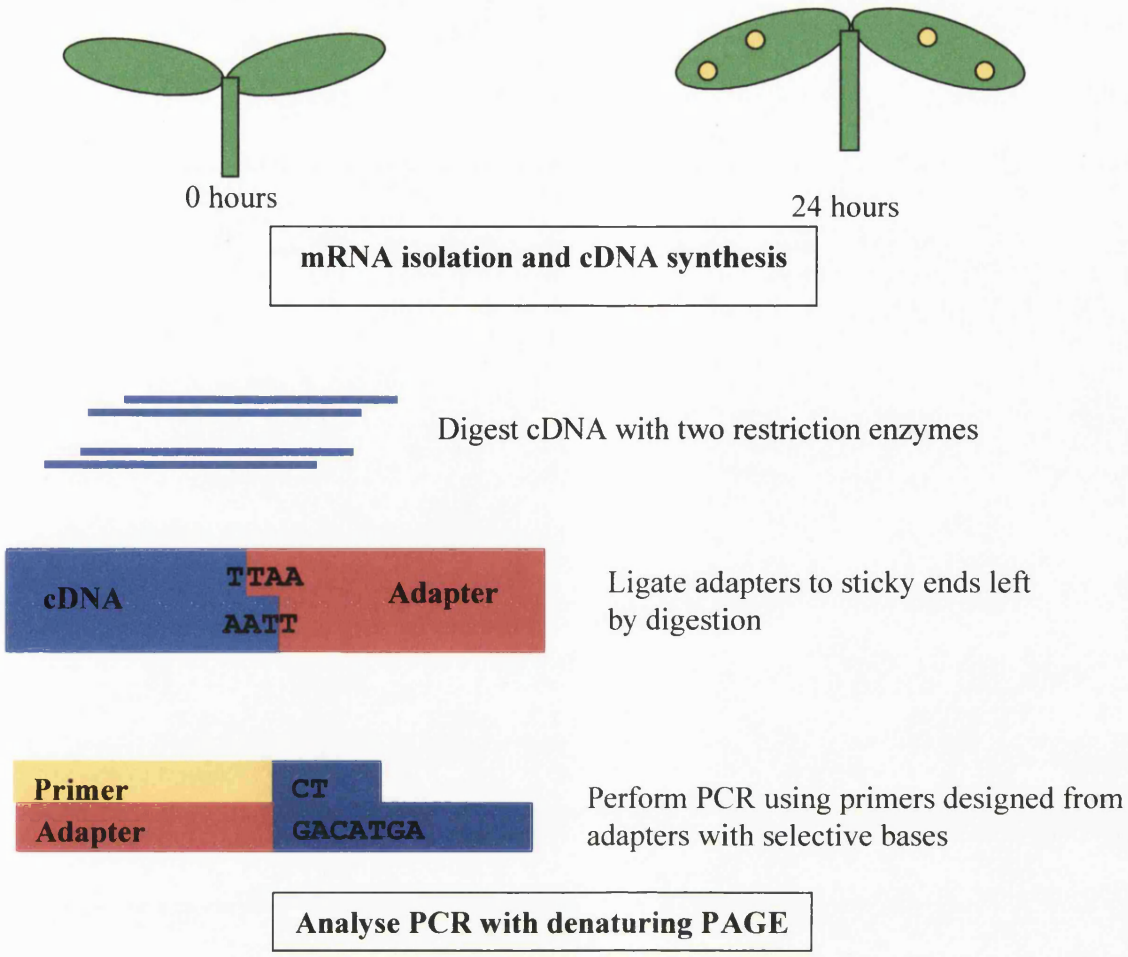


Figure 6.1 cDNA-AFLP
RNA is extracted from plants 0 and 24 hours after infection with a pathogen. cDNA is made and digested with two restriction enzymes, generally a rare cutter (in this study *Apo* I) and a frequent cutter (in this study *Mse* I). Adapters are then ligated to the sticky ends left by the digestion. The adapter provides the majority of the binding site for the primer and allows PCR to be performed at higher temperatures than for conventional differential display. Selective bases added to the ends of the primers are used to amplify a portion of the digested cDNA population. PCR reactions from the two (or more) treatments are then run out side by side on a denaturing polyacrylamide gel. Bands present in one treatment but not in the other can be cut out of the gel, amplified and sequenced.

function (Durrant *et al.,* 2000). Genes induced in *Chenopodium amaranticolor* plants hypersensitively responding to viral infection have been isolated by cDNA-AFLP (Cooper, 2001). The genes were similar to those involved in defence signalling, secondary metabolism and R genes from other plants (Cooper, 2001). In addition a gene with no known similarity in the database was increased 200 fold in response to two types of virus (Cooper, 2001). cDNA-AFLP has also been used to isolate genes involved in dehydration in almond (Campalans *et al.*, 2001), *Ageratum conyzoides* responding to *Agrobacterium tumefaciens* (Ditt *et al.*, 2001) and maize responding to *Bipolaris maydis* (Gao *et al.*, 2001).

Two studies have used cDNA-AFLP to investigate cassava. Suárez and co-workers (2000) elucidated differences between the two cassava cultivars used to create a molecular genetic map of cassava using cDNA-AFLP (Suárez *et al.*, 2000). Over 500 transcript derived fragments (TDFs) unique to either parent were detected, some of which have been incorporated into the molecular genetic map (Suárez *et al.*, 2000). cDNA-AFLP has also been used to identify genes involved in postharvest physiological deterioration (PPD) in cassava tubers. Of 6,000 TDFs screened 70 were differentially expressed during PPD (Huang *et al.*, 2001). Genes involved in oxygen stress, carbohydrate metabolism, protein metabolism and phenolic biosynthesis as well as a large proportion with no known function were isolated (Huang *et al.*, 2001).

The aim of this chapter is to elucidate some of the genes induced in cassava leaves in response to the incompatible pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*). This interaction was chosen because of the slow and incomplete expression of "resistant" cultivars of cassava to *Xam* (Chapter 3, this thesis).

6.2 Materials and Methods

6.2.1 Extraction of RNA

RNA was isolated from cassava leaves essentially as described by Leach *et al.* (1998). A cassava leaf was ground to a fine powder under liquid nitrogen. Frozen powder was transferred to a 50ml centrifuge tube containing 10ml TLES (100mM Tris-HCl, 100mM LiCl, 10mM EDTA, 1% (w/v) SDS) and 10ml aqueous saturated phenol and vortexed for 30 sec before the addition of 10ml chloroform: isoamyl alcohol (24:1 (v/v)). The mixture was vortexed for another 30 s and at that stage could be left on the bench for several minutes whilst other samples were prepared. Phases were separated by centrifugation at 5000g for 5 min. The aqueous layer was removed to a fresh 50ml tube containing 15ml phenol: chloroform: isoamyl alcohol (25:24:1 (v/v/v)) vortexed for 30s and centrifuged as above. Phenol: chloroform: isoamyl alcohol extractions were performed as above until no macromolecules were visible at the interface between the two phases. RNA was precipitated from the aqueous phase with the addition of an equal volume of 4.0M LiCl and incubation at 4°C overnight. RNA was pelleted by centrifugation for 30 min at 10,000g at 4°C. RNA was dissolved in 1 ml DEPC treated MilliQ water (Sambrook *et al.*, 1989), precipitated with 100µl 3.0M sodium acetate and 2.5ml 100% ethanol and collected by centrifugation at 10,000g for 20 min. RNA was washed in 70% ethanol and dissolved in 500µl DEPC treated MilliQ water.

6.2.2 Northern Blots

RNA loading buffer for one northern (at least 30 samples) was made by combining 620µl formamide, 186µl formaldehyde (37% (w/v)) and 155µl 10xMEN (200mM MOPS, 50mM sodium acetate, 20mM EDTA, pH 7.0) with a spot of 10mg ml^{-1} ethidium bromide. RNA was denatured by the addition of 31µl of loading buffer to 20 µg of total RNA in 20µl of H_2O and incubating at 65°C for 15 min. Denatured RNA was separated on a 1.5% agarose gel containing 0.06% formaldehyde in 1xMEN buffer. Gels were run at 100 volts for several h before viewing under UV light. RNA was transferred to Hybond XL (Amersham Biosciences) as described by the manufactures and fixed using an optimised UV crosslinking procedure (Sambrook *et al.*, 1989).

DNA probes were labelled with [α-^{32}P] dCTP (ICN) using an oligolabelling kit (Amersham Biosciences) as manufacturer's instructions. Unincorporated nucleotides were removed using a Sephadex G-50 (Amersham Biosciences) spin column (Sambrook *et al.* 1989). Hybridization was performed using the protocol of Church and Gilbert (1984). Membranes were washed in 0.1% SDS and 0.1xSSC twice for 15 min at 65°C before exposure to Kodak X-OMAT film.

6.2.3 Synthesis of cDNA

cDNA was synthesised from total RNA using the SMART cDNA synthesis kit (BD Biosciences Clontech, Crawley, Oxford, UK) essentially as described by the manufacturers. First strand cDNA was synthesised from 1μg of total RNA using 7μM of the SMART and cDNA synthesis oligonucleotides provided. After denaturing at 70°C for 2 min the RNA and primers were incubated with 150 units Superscript II MMLV reverse transcriptase (Invitrogen) in 1x Reaction buffer in a total volume of 7μl at 42°C for 1 h.

Second strand cDNA synthesis and amplification was carried out by PCR. Two microlitres of first strand reaction was amplified with the PCR primers provided and the Advantage 2 PCR kit (BD Biosciences Clontech) in a total volume of 100μl. PCR was carried out in a PE 9600 thermal cycler (Applied Biosystems); cycling conditions were denaturation at 95°C for 1 min followed by 17 cycles of denaturation at 95°C for 6 s, annealing at 65°C for 6 s and extension at 68°C for 6 min. Five μl of the reaction was checked on a 1xTBE gel. Proteins were removed from the cDNA by a phenol/chloroform extraction followed by ethanol precipitation. cDNA was redissolved in 100μl H_2O and unincorporated nucleotides and oligonucleotides were removed with a NucleoTrap purification kit (BD Biosciences Clontech).

6.2.4 Template Preparation

Template preparation was essentially as detailed in Vos *et al.* (1995) and Bachem *et al.* (1996) using adapters described by Durrant *et al.* (2000). One microgram of cDNA was digested with 16 units of *Apo* I (New England Biolabs) in 1x reaction buffer with 0.1mg ml^{-1} BSA in a total volume of 80μl at 50°C for 2 h. Sixteen units of *Mse* I (New England Biolabs) were then added, the volume made up to 100μl with 1x reaction buffer and the reaction incubated at 37°C for 2 h.

Apo I and *Mse* I adapters were made by boiling a solution of two oligonucleotides for 2 min and allowing them to cool slowly. *Apo* I adapters were made from WD20 and WD50, *Mse* I adapters were made from WD47 and WD48 (see appendix 3 for primer details). Adapters were ligated to the digested cDNA by the addition of 2μl One Phor all buffer (Stratagene), 0.2mM rATP, 5pmol *Apo* I adapter, 50pmol *Mse* I adapter and 8 units T4 DNA ligase (Stratagene) in a total volume of 20μl to the digested cDNA and incubating at 37°C overnight.

6.2.5 Amplified Fragment Length Polymorphism (AFLP)

AFLP was essentially as detailed in Bachem *et al.* (1996) with primers from Durrant *et al.* (2000) (see appendix 3 for primer details).

The AFLP template was preamplified using WD16 and WD19 in a PCR reaction containing 20 pmol of each primer, 0.25mM dNTPs, 1 unit of Taq DNA polymerase (Promega), 20µl of the template in 1x reaction buffer in a total volume of 50µl. Cycling conditions were denaturation at 95°C for 30 s followed by 20 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 30 s and extension at 72°C for 60 s. Three microlitres of the reaction was analysed on a 1xTBE gel.

AFLP reactions were carried out with *Mse* I primers with two selective bases and *Apo* I primers with one selective base. *Apo* I primers (150ng) were radiolabelled with 2.5µl [γ ^{33}P]-ATP (10 µCi µl^{-1}) using 1 unit T4 polynucleotide kinase (Invitrogen) in 1x reaction buffer in a total volume of 15µl at 37°C for 1 h. This provided enough labelled primer for 30 AFLP reactions.

Selective amplification was conducted in 10µl volumes containing 5ng labelled *Apo* I primer, 30ng *Mse*I primer, 0.25mM dNTPs, 2.5mM $MgCl_2$, 1 unit of Taq, 0.125µl of the preamplified template in 1x reaction buffer. Touchdown PCR was carried out using the following conditions: denaturing at 95°C for 30 s followed by 13 cycles of denaturation at 95°C for 30 s, annealing at 65°C minus 0.7°C per cycle for 30 s, extension 72°C for 1 min, followed by 22 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min.

6.2.6 Polyacrylamide Gel Electrophoresis (PAGE) analysis of AFLP reactions.

Denaturing PAGE was carried out using a Bio-Rad DNA sequencing apparatus. For each gel 90ml 5% acrylamide (19 parts acrylamide to 1 part bis-acrylamide) in 45% w/v urea, 10ml 10x TBE, 40µl TEMED and 400µl 10% ammonium persulphate were mixed and poured into the gel setting apparatus. The gel was left to polymerise for at least 1h. The gel was pre-run in 1xTBE at 80 watts for 1 h to warm the gel prior to the addition of samples.

Samples were prepared by the addition of an equal volume of loading buffer (98% formamide, 10mM EDTA pH 8.0, bromophenol blue, xylene cyanol) and heating to 100°C for 2 min to denature the DNA then cooling on ice.

Samples were loaded on the gel and run at 80 watts (constant wattage) for approximately 3 h until the xylene cyanol had migrated three quarters of the way down the gel (xylene cyanol migrates with DNA of approximately 140bp in a 5% denaturing gel). Gels were stuck to Whatman No 1 filter paper and dried at 80°C for 2h in a gel drier (Bio-Rad). Gels were exposed to Biomax MR film (Eastman Kodak) for 24h.

6.2.7 Isolation and sequencing of Transcript Derived Fragments (TDFs)

Films were placed on top of the dried gel and aligned. Bands showing differential expression were cut out and incubated in 60µl H_2O for 1 h at –20°C followed by 2h at 65°C. TDFs were amplified by PCR using *Apo* I and *Mse* I primers with no selective bases. Ten microlitres of eluted TDF was amplified in 50µl PCR reactions. PCR products were agarose gel purified using the Qiaquick gel extraction kit (Qiagen) and eluted in ddH_2O to ensure no primers were present that may have interfered with sequencing the PCR products. 5ng of PCR product were sequenced using an ABI automated sequencer. All TDFs were sequenced using the *Apo* I primer with no selective bases. TDFs that failed to give any sequence data were re-amplified from the eluted TDFs using the selective primers that gave rise to the original TDF. These re-amplified products were gel purified and sequenced with the *Apo* I primer with the appropriate selective base. TDFs that gave sequence chromatograms that indicated a double product were blunt ended with T4 DNA polymerase and ligated into pBluescript previously cut with *Eco*RV and dephosphorylated. The resulting plasmids were then sequenced using the T7 sequencing primer.

6.3 Results

The hypersensitive response was induced on cassava leaves by infiltration with $1x10^8$ cfu ml^{-1} *Pseudomonas syringae* pv. *tomato* (*Pst*). After 48 h, areas infiltrated with *Pst* were necrotic, in comparison with water-inoculated controls, which showed no symptoms. Using existing probes, β 1-3 glucanase and phenylalanine ammonia lyase transcripts were shown to have increased 24 h after infection with *Pst* but not after infiltration with water (figure 6.2).

6.3.1 Detection of Differentially Expressed Genes using cDNA-AFLP

RNA was extracted from cassava leaves 0 and 24h post infection with *Pst*. The SMART cDNA synthesis kit successfully converted total RNA to full length cDNAs with a range of sizes between 5kb and 0.5kb (figure 6.3). This range is similar to that quoted in the kit instructions for mammalian total RNA (10kb to 0.5kb). No bright bands, indicative of abundant cDNAs, were visible, probably because of the complexity of the original RNA samples. After template preparation the preamplified template showed a size distribution of between 1kb and 0.1kb with the vast majority of template present at around 0.3kb (figure 6.4).

A total of 36 different primer combinations were used (28% of the total possible combinations of 128) on a total of 2 templates, and all reactions were replicated. When the reactions were run out on denaturing PAGE discrete bands were seen on the autorads (figure 6.5). Approximately 100 transcript-derived fragments (TDFs) were seen for each primer combination, therefore around 7,000 bands were inspected for differential expression. The vast majority of bands were common to both time points and the replicates showed good agreement. 78 TDFs were only present at one time point; 75% were present in the 24 hour time point but not in 0 hour samples (up-regulated TDFs) and 25% were absent from the 24 hour time point (down-regulated TDFs). All excised TDFs amplified well by PCR showing single bands (figure 6.6) indicating that excision had been accurate. 63% of amplified TDFs gave usable data on the first sequencing run. Subsequent sequencing runs resulted in good quality data for over 90% of differentially expressed TDFs. The majority of the sequencing reactions gave reads covering the whole length of the TDF, determined by detecting the *Mse* I primer at the 3 prime end of the sequence. The average length of the TDF sequences was 270 base pairs with a range from 122 to 511 base pairs. Differentially expressed TDFs were named Bacterial Cassava AFLPs (BCA) and numbered sequentially. Sequences were compared to the Genbank database using BlastX to see if they had similarity to known proteins. TDFs with similarities to a number of characterised genes were detected (Table 6.1). Based on resemblance to genes in the

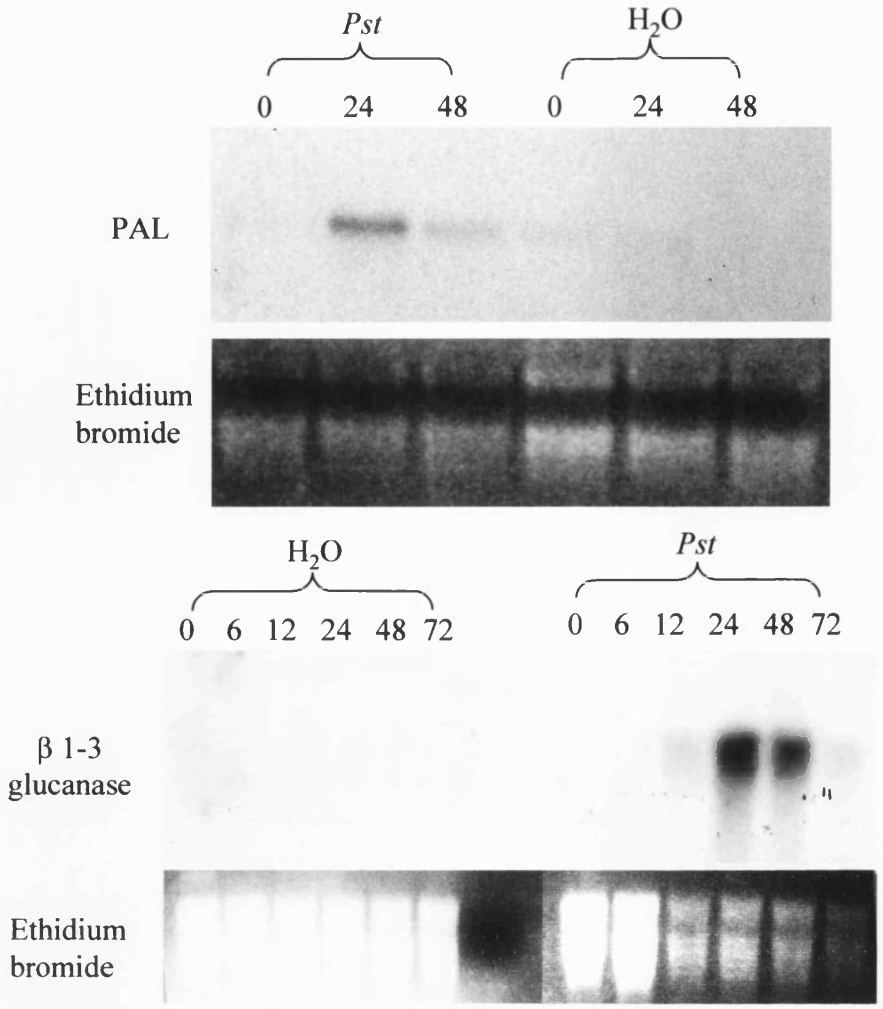


Figure 6.2 Northerns of cassava leaf RNA showing differential expression of two PR transcripts.
20μg of total RNA extracted at different time points from cassava leaves infiltrated either with a $1x10^8$ cfu ml^{-1} suspension of *Pst* (*Pst*) or water (H_2O) was fractionated on a formaldehyde gel and transferred to Hybond N+. cDNAs encoding a cassava phenylalanine ammonia lyase (PAL) and cassava β 1-3 glucanase (both isolated by Dr. Y. Han at the University of Bath) were used to probe the northerns. An up-regulation of PAL transcript can be seen 24 h after infection with *Pst*, a low level of basal expression can also be seen in the water controls. An up-regulation of glucanase transcripts can be seen after 12 h before peaking at 24 h, no expression can be seen in the water controls. Loading is shown with ethidium bromide stained gels pictured prior to nucleic acid transfer (N.B. the loading of the *Pst* infected northern used to test for glucanase expression appears to decrease across the gel, this is due to limitations of the gel documentation system used to take this image. When viewed by the naked eye all lanes showed equal loading)

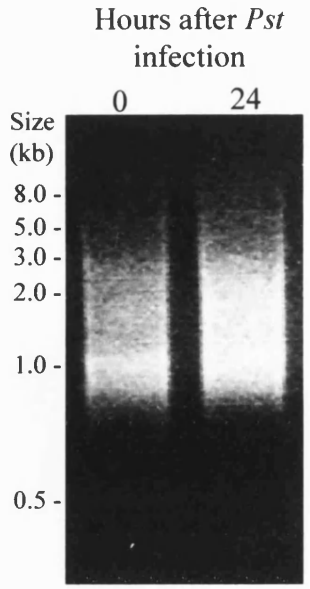


Figure 6.3 cDNA from *Pst* infected cassava leaves.
Total RNA was used as a template for cDNA synthesis using the SMART cDNA synthesis kit (Clonetech). All cDNA synthesised using this system is full length. The resulting cDNA was analysed on an agarose gel showing a range of sizes from 0.5kb to over 5.0kb.

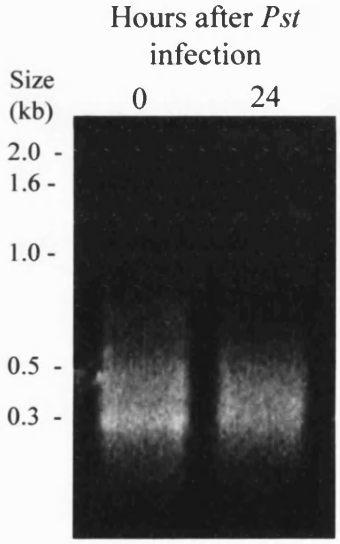


Figure 6.4 Pre-amplified AFLP template.
cDNA was digested with *Mse* I and *Apo* I and adapters were ligated onto the digested cDNA. Primers with no selective bases were used to amplify the cDNA prior to the AFLP reaction. The gel above shows an aliquot of the pre-amplified template indicating that restriction digestion and ligations had been successful. Note the dramatic reduction in size compared with cDNA (figure 6.3)

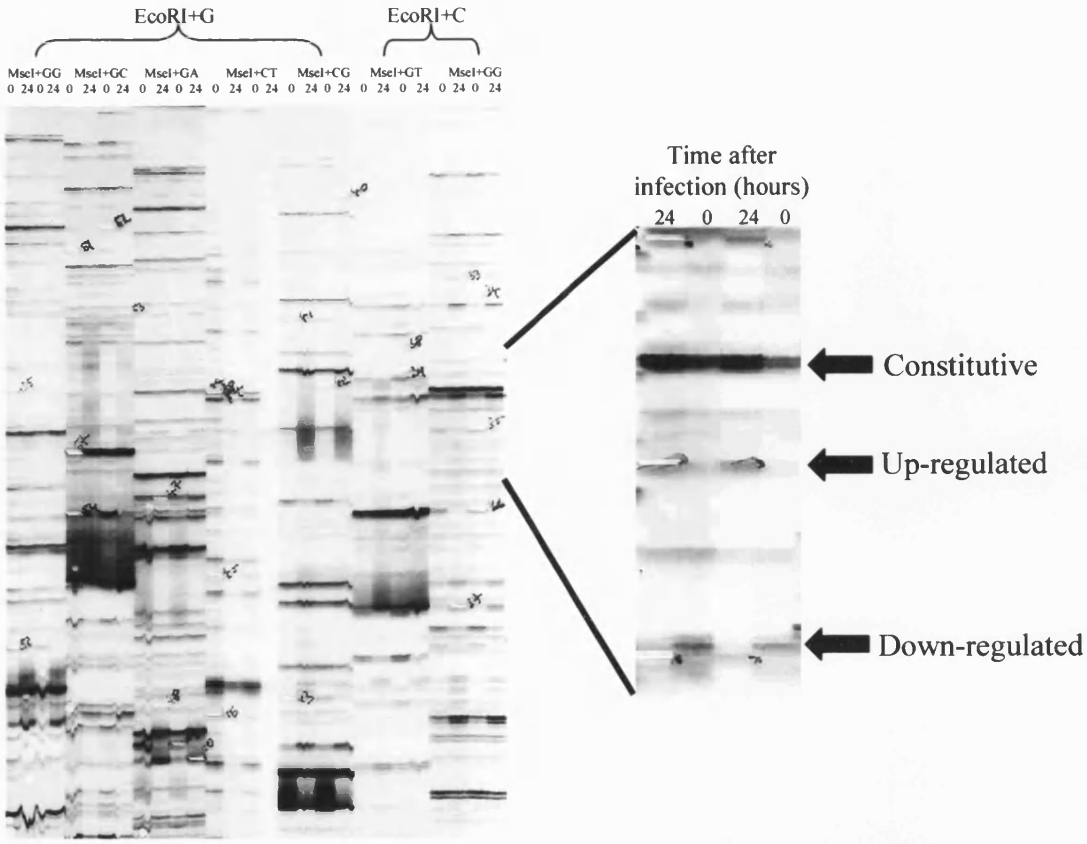


Figure 6.5 Autoradiograph of AFLP gel.
AFLP reactions from *Pst* infected cassava at time 0 and 24 hours post infection were run along side each other and subjected to autoradiography. Each AFLP reaction was repeated. The main autoradiograph (on the left) shows the primer combinations used in the AFLP reactions, the *Eco* RI primers prime the *Apo* I adapter. The close-up of the autoradiograph (on the right) shows the three classes of TDFs (transcript derived fragments) found; TDFs from constitutively expressed genes (constitutive), TDFs from genes induced in response to *Pst* (up-regulated) and TDFs from genes repressed in response to *Pst* (down-regulated). Up- and down- regulated TDFs were excised from the gel (using the autoradiograph as a template, the areas cut away can be seen on the left hand lanes of the close-up) and amplified using PCR (see figure 6.6).

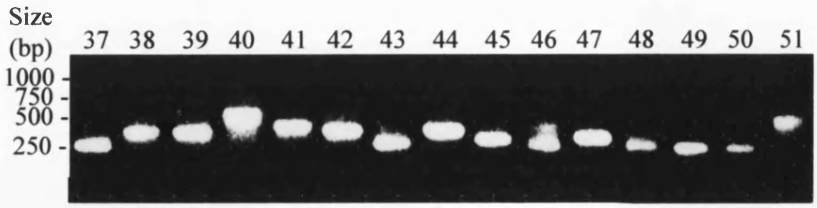


Figure 6.6 Amplified TDFs.
TDFs were excised from the AFLP gel and eluted in 60µl water. 10µl of eluted TDF was used as a template in a 50µl PCR reaction. Gel photo above shows 5µl of PCR products from BCA37-51 run on an agarose gel. All TDFs amplified successfully and only showed single bands indicating that the excision had been accurate. Amplified TDFs were subsequently gel purified and sequenced.

Fragment Number	Length (bp)	Regulation	Similarity	Blast E value
BCA1	224	Up	Calcium-transporting ATPase from *A. thaliana* (AP001300)	2.00E-18
BCA2	235	Up	Protein kinase from Rice (AP001550)	3.00E-14
BCA3	189	Up	Juvenile hormone resistance protein from *Drosophila* (AF034859)	2.1
BCA5	484	Down	Flavonol 3-O-gluxosyltransferase from Cassava (X77463)	2.00E-70
BCA7	232	Up	Predicted protein from *A. thaliana* genome sequence (AB026636)	8.00E-22
BCA9	275	Up	Calmodulin-like protein *A. thaliana* (AL356014)	0.21
BCA14	404	Up	NADP-dependent malic enzyme from Poplar (X56233)	7.00E-39
BCA15	262	Up	Catalase from Cassava (AF170272)	7.00E-33
BCA20	385	Up	Proteasome precursor from *A. thaliana* (AL021633)	2.00E-46
BCA22	511	Up	Predicted protein from *A. thaliana* genome sequence (AC007138)	1.00E-50
BCA23	448	Up	Beta-1,3 glucanase from Pea (AJ251646)	1.00E-40
BCA25	391	Up	Predicted protein from *A. thaliana* genome sequence (AC007454)	2.00E-06
BCA26	264	Up	2-oxoacid dependent oxygenase ARRO-1 from Apple (AJ225045)	9.00E-33
BCA28	430	Up	Metalloproteinase from Cucumber (AJ133371)	9.00E-27
BCA31	331	Up	Nucleotide pyrophosphatase-like protein from *A. thaliana* (AL079344)	6.00E-17
BCA34	344	Up	DAHP synthase from *Morinda citrifolia* (MCY14797)	2.00E-49
BCA35	258	Up	Predicted integral membrane protein from *A. thaliana* genome sequence (AC006284)	0.066
BCA36	219	Up	DNA-binding protein 4 from Tobacco (AF193771)	3.00E-12
BCA37	196	Down	Glycine hydroxymethyltransferase from *Flaveria pringlei* (Z25860)	7.00E-25
BCA38	315	Up	*A. thaliana* protein similar to cysteine desulfurase (AC006932)	3.00E-12
BCA39	291	Up	Predicted protein from *A. thaliana* genome sequence (AC010927)	0.002
BCA40	456	Down	No related sequences in the database	
BCA41	328	Up	S-adenosyl-L-homocysteine hydrolase from *A. thaliana* (AF059581)	1.00E-43
BCA42	279	Up	Beta-glucosidases from *A. thaliana* (AC009525)	1.00E-27
BCA43	164	Up	Predicted protein from *A. thaliana* genome sequence (AC004122)	2.00E-10
BCA44	272	Up	Cinnamoyl CoA reductase from Poplar (AF217958)	5.00E-37
BCA45	202	Up	Putative cytochrome P450 from Rice (AP002093)	2.00E-23

Fragment Number	Length (bp)	Regulation	Similarity	Blast E value
BCA47	227	Down	ADP-glucose pyrophosphorylase from Fava bean (X76941)	2.00E-28
BCA49	147	Up	No related sequences in the database	
BCA50	145	Down	CONSTANS-like B-box zinc finger protein from *A. thaliana* (AC006585)	1.00E-14
BCA51	403	Up	Dihydroflavonol reductase from Apple (AF117268)	4.00E-55
BCA52	397	Up	Predicted protein from *A. thaliana* genome sequence (AL132977)	5.00E-10
BCA54	198	Up	10 kDa chaperonin from *Brassica napus* (U65890)	2.00E-19
BCA56	187	Up	No related sequences in the database	
BCA57	270	Constutive	RUBISCO small chain from Cassava (AF101231)	2.00E-44
BCA59	212	Up	ACC oxidase from *Prunus armeniaca* (AF026793)	3.00E-22
BCA64	300	Up	p23 co-chaperone from *A. thaliana* (AJ297951)	4.00E-05
BCA74	183	Up	Peroxidase from *Populus kitakamiensis* (D30653)	2.00E-10
BCA75	288	Down	Carbonic anhydrase from *Vigna radiata* (AF139464)	2.00E-05
BCA76	160	Up	26S proteasome ATPase subunit from Spinach (D86121)	8.00E-21

Table 6.1 Similarities to known genes of TDFs
The similarity of TDFs to genes in the database was determined using BlastX (Altschul *et al.*, 1997) where the nucleotide sequence is converted to protein sequence (six possible combinations) and compared to protein sequences and translated nucleic acid sequences. Length = the length of usable sequence data obtained for each TDF. Similarity = The sequence from the database with highest similarity to the TDF as determined by BlastX.E-value = the e-value of the TDF compared to the gene shown in similarity.

databases the up-regulated TDFs could be placed into one of eight groups (figure 6.7). The largest grouping was of genes involved in protein synthesis and processing with nine members, followed by genes involved in photosynthesis and *A. thaliana* genes of no known function (both with eight members). Seven TDFs were similar to genes involved in secondary metabolism and six were involved in local and systemic signalling. Five genes were involved in defence responses and five in primary metabolism. Five TDFs had either very low (e value ≥ 0.01) or no similarity to anything in the database.

Down-regulated TDFs could be placed into one of six groups based on similarity to genes of known function (figure 6.7). Five TDFs were similar to genes involved in photosynthesis, four to ubiquitins and four to genes of primary metabolism. Other groups of secondary metabolism, unknown function and no homology all had one member.

6.3.2 Confirmation of differential expression

In order to draw any conclusions about the role of TDFs in the cassava defence response it was first necessary to confirm their differential expression. Initially 31 TDFs were chosen for further analysis either because they were similar to genes involved in the defence response in other systems or because they were similar to genes with unknown function.

TDFs were radiolabelled and used to probe northern blots; however the majority showed high levels of non-specific binding or highlighted multiple bands (figure 6.8 and Table 6.2). This may have been due to the short length of the TDFs or possibly because the TDFs encoded for the conserved regions of genes. However where the probes bound multiple bands it was often possible to see differential expression of one band (Table 6.2).

Of 31 northerns, eight (BCA3, BCA7, BCA5, BCA23, BCA28, BCA39, BCA40 and BCA51) showed an expression pattern similar to that predicted by the AFLP gels. Three showed the opposite expression pattern and two had variable expression levels that did not appear to be determined by the HR. Seven showed constitutive expression (Table 6.2). Seven northerns showed non-specific binding to all RNA and four had very low signal strength. Of the eight northerns agreeing with the AFLP gel, only BCA28 showed hybridisation to one band and a definite up-regulation of expression in response to *Pst* (figure 6.9).

RT-PCR was used as another test of differential expression on 10 of the 31 TDFs. Five (BCA2, BCA9, BCA22, BCA23 and BCA36) showed differential expression (figure 6.10). The rest showed constitutive expression using RT-PCR (figure 6.10).

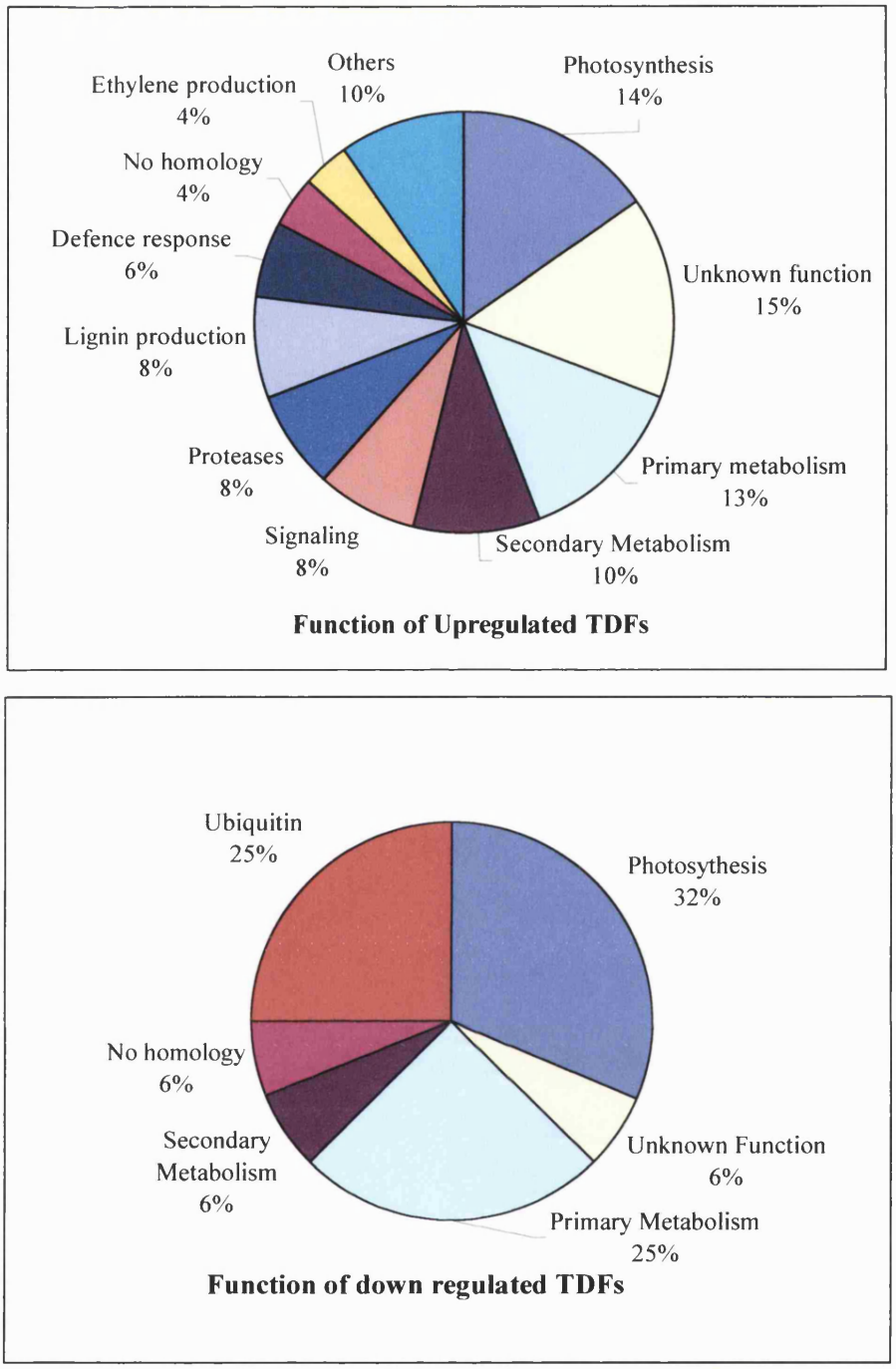


Figure 6.7 Hypothesised function of differentially expressed TDFs.
TDFs were grouped according to their similarity to genes of known function when compared to the database with BlastX.

Fragment	Highest similarity to	AFLP regulation	Northern regulation	Northern blot H_2O	Northern blot *Pst*
BCA2	Protein kinase	Up	Down		
BCA3	Juvenile hormone resistance protein from *Drosophila*	Up	Up-HR		
BCA5	Flavonol 3-O-glucosyltransferase	Down	Down-HR		
BCA7	Predicted protein from *A. thaliana* genome sequence	Up	Up-HR		
BCA9	Calmodulin-like protein	Up	NSB		
BCA13	Glycine-rich protein	Up	Variable		
BCA14	NADP-dependent malic enzyme	Up	Constitutive		
BCA15	Catalase	Up	NSB		
BCA20	Proteasome precursor	Up	NSB		
BCA22	Predicted protein from *A. thaliana* genome sequence	Up	Constitutive		
BCA23	Beta-1,3 glucanase	Up	Up-HR		
BCA25	Predicted protein from *A. thaliana* genome sequence		NSB		
BCA28	Metalloproteinase	Up	Up-HR		
BCA31	Nucleotide pyrophosphatase	Up	Variable		
BCA35	Predicted integral membrane protein	Up	Down		
BCA36	DNA-binding protein	Up	Constitutive		
BCA37	Glycine hydroxymethyltransferase	Down	NSB		
BCA39	Predicted protein from *A. thaliana* genome sequence	Up	Up-HR		
BCA40	No related sequences in the database	Down	Down		

BCA41	S-adenosyl-L-homocysteine hydrolase	Up	Constitutive	
BCA42	Beta-glucosidases	Up	NSB	
BCA43	Predicted protein from *A. thaliana* genome sequence	Up	LSS	
BCA44	Cinnamoyl CoA reductase	Up	LSS	
BCA49	No related sequences in the database	Up	LSS	
BCA51	Dihydroflavonol reductase	Up	Up	
BCA52	Predicted protein from *A. thaliana*	Up	Constitutive	
BCA56	No related sequences in the database	Up	Down	
BCA59	ACC oxidase	Up	Constitutive	
BCA70	Peroxidase	Up	LSS	
BCA74	Peroxidase	Up	Constitutive	
BCA75	Carbonic anhydrase	Down	NSB	

Table 6.2 Expression analysis of differentially expressed TDFs.
Purified TDFs were used as probes for Northern blots, autoradiographs are shown above. 20μg of total RNA from 0, 6, 12, 24, 48 and 72 h after infiltration with either water (H_2O) or *Ps. syringae* pv. *tomato* (*Pst*). Time points are from left (0 hours) to right (72 hours). AFLP regulation is as determined by the original AFLP gel; Up-HR = up-regulation for *Pst* inoculated but not water control leaves, Down-HR = down-regulation in *Pst* infected leaves but not water control, Up = up-regulation for both *Pst* inoculated and water control leaves, Down = down-regulation for both *Pst* infected and control leaves, Variable = changing expression levels over time, Constitutive = no changing in expression on infection NSB = non-specific binding, LSS = Low signal strength. Note that because of the unreliability of TDFs as Northern probes (see figure 6.8) extreme caution must be taken when interpreting results from this table.

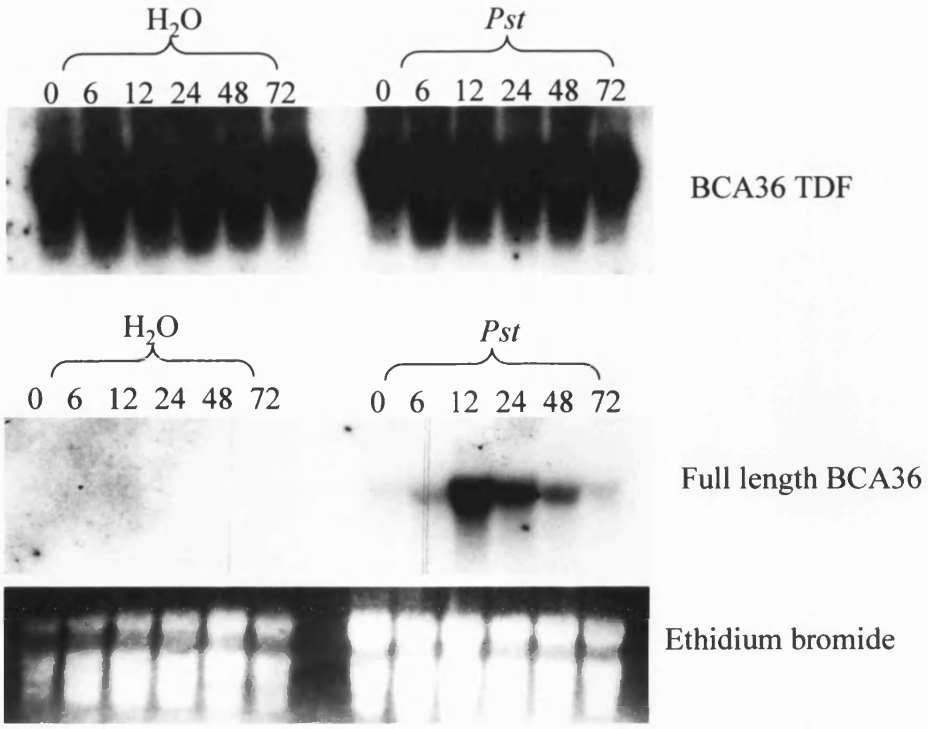


Figure 6.8 Non-specific binding
The top autoradiograph shows a northern blot of RNA from infected cassava leaves probed with the 219bp TDF BCA36. A large amount of non-specific binding is seen. In contrast the bottom panel shows a northern probed with a full length clone of BCA36 and shows up regulation of transcript in response to *Pst* and no up regulation in water controls (H_2O). This figure graphically illustrates the problems associated with using TDFs as probes for northerns. The lower panel shows equal loading of the gel.

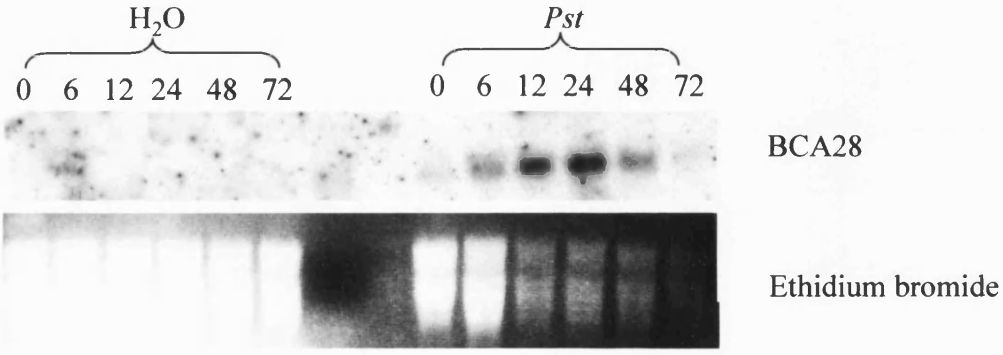
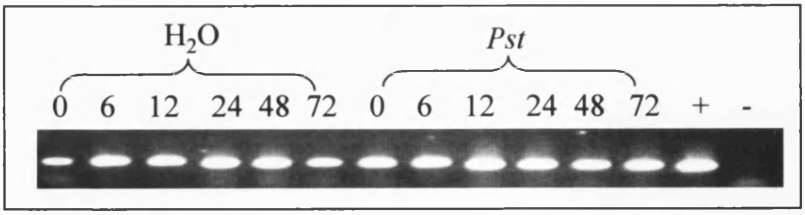
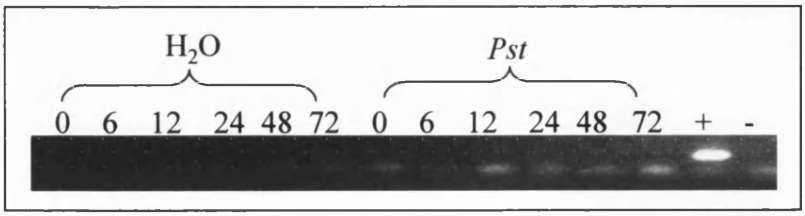


Figure 6.9 Northern blot of BCA28
BCA28 was a relatively long TDF (430bp long) with high similarity to a matrix metalloprotease from cucumber. Possibly because of its length BCA28 was the only TDF to hybridise to a single band on a northern blot. Transcripts are up regulated 6 h after infection with *Pst*, no transcripts were detected in the water (H_2O) controls. Loading is shown with ethidium bromide stained gels pictured prior to nucleic acid transfer (N.B. the loading of the *Pst* infected northern used to test for BCA28 expression appears to decrease across the gel, this is due to limitations of the gel documentation system used to take this image. When viewed by the naked eye all lanes showed equal loading)



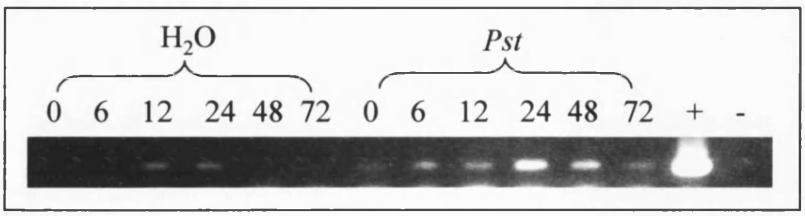
Constitutive RT-PCR

BCA14 appears to be from an NADP-dependent malic enzyme. Northern blot analysis showed constitutive expression. RT-PCR confirmed this constitutive expression indicating that BCA14 is not induced in response to *Pst.* The size of the PCR product is ca. 400bp.



No transcript detected RT-PCR

BCA20 is similar to Proteasome precursor. Northern blots probed with BCA20 showed high levels of non-specific binding. RT-PCR failed to amplify transcripts indicating that the gene is expressed at very low levels. The size of the expected PCR product is ca. 380bp and a positive control (+) reaction with the original TDF as template indicates that the PCR conditions and primer design are adequate. The small, weak band present in some lanes is also present in the negative control (-) and may result from primer dimers.



Differentially expressed RT-PCR

BCA22 is similar to an *Arabidopsis* gene of no known function. Northern blot analysis of the TDF appeared to show constitutive expression. RT-PCR analysis shows that the transcript is up-regulated on infection with *Pst* with levels peaking at around 24h post infection. Small amounts of transcript are also present 12 and 24h after infection with water but a more sustained and rapid increase in transcript level is seen after *Pst* infection. The size of the PCR product is ca. 500bp.

Figure 6.10 Representative RT-PCR reactions. RT-PCR reactions grouped into three types as shown. All reactions used the same template, time in hours after infection and the treatment is indicated at the top of the gel. + = positive control reaction with original TDF as template - = negative control with no template

6.3.3 Isolation of full length clones

Six of the TDFs with confirmed differential expression were used to isolate cDNA clones. The selection of these clones was based on confirmation of differential expression either by northerns (BCA28 and BCA40) or RT-PCR (BCA2, BCA9 and BCA22). BCA36 was also selected because of high similarity to the WRKY transcription factors.

A cDNA library was constructed from cassava leaves 24h post infection with *Pst*. Ten micrograms of mRNA was isolated from 1mg total RNA using the PolyATract system (Promega). Five micrograms was then used to create a cDNA library in the Uni-ZAP XR λ vector (Stratagene). All controls recommended by the manufacturers were included and resulted in a primary library with a phage titre of $1x10^6$ pfu with less than 1% containing no insert. The primary library was amplified and resulted in a secondary library with a titre of $2.6x10^{11}$ pfu ml^{-1}. Five plates of 50,000 plaques were then screened for each TDF. Positive plaques were subjected to a second round of screening before plasmid excision with the ExAssist helper phage. Antibiotic resistant colonies were screened for plasmids containing the gene of interest by colony PCR. Plasmid minipreps were performed on positive colonies and the resulting plasmids were sequenced.

Sequences were assembled using the GCG Wisconsin package of programs. The cDNA clones all showed good alignment with the original TDFs (table 6.3).

The cDNAs were translated *in silico* in all six frames and the longest open reading frame selected for further study. Predicted protein size was calculated using the SAPS program (table 6.3). Some cDNAs were used to probe northerns and gave much better results than the corresponding TDFs (figure 6.9).

BCA2 encodes a putative protein kinase. The initial TDF northern appeared to show a down-regulation of expression 6 h after infiltration with either water or *Pst* (table 6.2). However the TDF bound to two bands on the northern. RT-PCR showed that BCA2 appeared to be rapidly expressed in response to wounding (figure 6.11). However the final northern blot with a full-length clone appeared to mimic the initial TDF northern, showing hybridisation to two bands and an apparent rapid down-regulation in response to both *Pst* and to water (figure 6.11).

BCA9 encodes a protein with a calcium-binding motif at the C-terminal end. The initial TDF northern showed very high levels of non-specific binding. RT-PCR analysis appeared to show a basal level or expression followed by rapid up-regulation in response to *Pst* and down-regulation in response to water infiltration (figure 6.12).

Clone	Size of cDNA	Predicted protein size	% identity to TDF
BCA2	1382	350aa, 39.9 kDa	90
BCA9	1712	161aa, 18.3 kDa	97
BCA22	1117	283aa, 31.5 kDa	97
BCA28	1167	102aa, 11.9 kDa	93
BCA36	999	220aa, 24.7 kDa	97
BCA40	630*	178aa, 18.9 kDa*	97*

Table 6.3 Length of clones isolated from cassava cDNA library. Clones were isolated from the cassava leaf cDNA library and sequenced using the T3 and T7 primers and internal primers. Vector DNA was removed and the length of cassava cDNA (including polyA tail) calculated. The sequence was translated *in silico* and the longest open reading frame used to calculate predicted protein length and size (Brendel *et al.*, 1992). * = incomplete sequencing of this gene, data is from available sequence

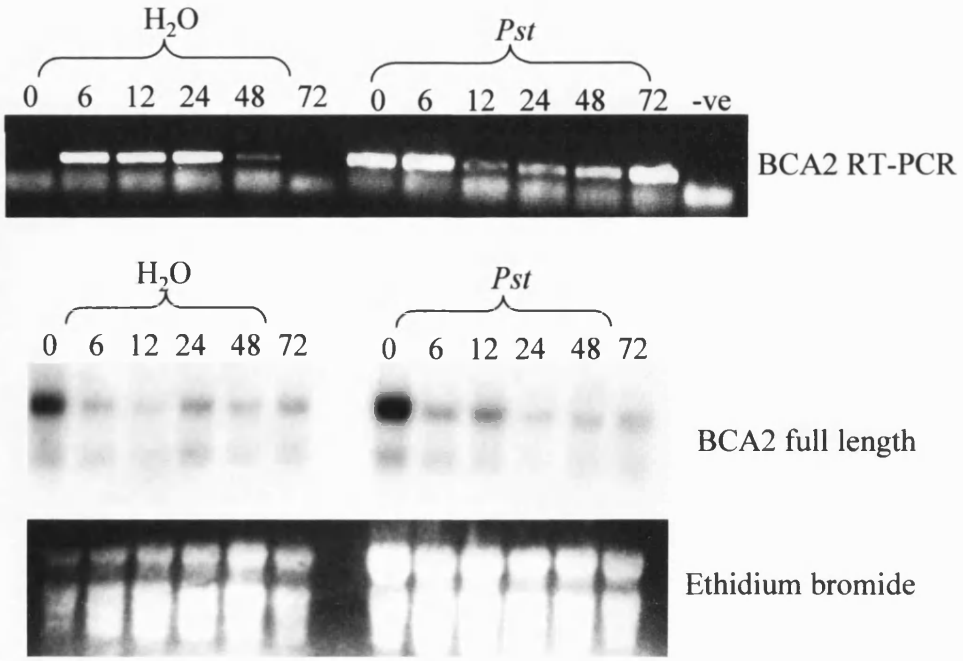


Figure 6.11 Expression patterns of BCA2
BCA2 encodes a putative protein kinase which was up-regulated in the AFLP reaction. However initial northerns indicated that it was down regulated (see table 6.2). RT-PCR proved inconclusive (see above) and a northern probed with a full length version of the gene was identical to the initial northern, hybridising to more than one band. In the light of this conflicting evidence no role can be assigned for BCA2. RT-PCR was conducted as detailed in figure 6.11. - = negative control with no template. Northerns were blots of 20μg total RNA probed with a full length version of BCA2, equal loading of the gel is shown by ethidium bromide staining.

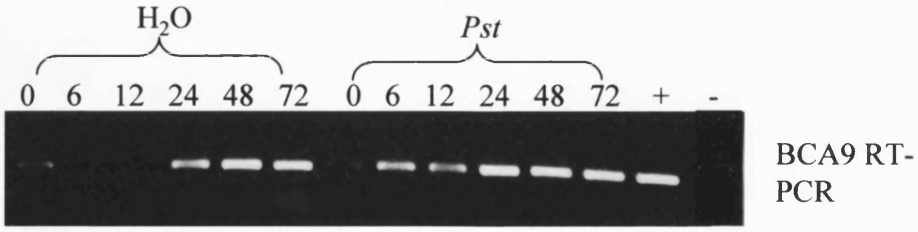


Figure 6.12 Expression of BCA9
BCA9 encodes a putative calcium binding protein. Whilst initial northerns (table 6.2) had a high level of non-specific binding RT-PCR indicates that BCA9 is up-regulated in response to infiltration. The up-regulation appears to be much more rapid in leaves infiltrated with *Pst* than those infiltrated with water (H_2O). RT-PCR was conducted as detailed in figure 6.10. + = positive control with TDF as template, - = negative control with no template.

BCA22 encodes a protein similar to an *A. thaliana* gene with no known function. It is also similar to the *tolB* gene from *E. coli*. The clone obtained for BCA22 appears to be about half the size of the *A. thaliana* protein. Initial northern analysis using the TDF as a probe showed constitutive expression (table 6.2). RT-PCR appeared to show up-regulation in response to *Pst* (figure 6.10).

BCA28 encodes a protein very similar to a matrix metalloprotease from cucumber. The initial northern showed up-regulation in response to infection with *Pst* but not in response to infiltration with water (figure 6.9).

BCA36 encodes a protein very similar to a WRKY transcription factor. Initial northern analysis showed constitutive expression. However a northern probed with a full length clone showed expression is up-regulated in response to infection with *Pst* but is not up-regulated in response to infiltration with water (figure 6.8).

BCA40 encodes a protein with no similarity to anything in the database. Initial northerns showed down regulation in response to both pathogen attack and infection with *Pst* (table 6.2).

6.4 Discussion

Resistance of cassava to bacterial blight is only partial and dependent on climatic, edaphic and geographic factors (Jorge *et al.,* 2001). In order to understand the potential induced defence mechanisms of cassava a system was needed that, unlike CBB, resulted in rapid and consistent resistance. The tomato pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) was shown to induce the hypersensitive response on cassava leaves. Necrosis within the infiltration site was visible within 24h. In this tissue β 1-3 glucanase and phenylalanine ammonia lyase transcripts were initially up-regulated 6 h after infiltration with a peak of expression 24 h after infiltration. The *Pst* -cassava interaction therefore provided a system to isolate defence-related genes from cassava that may provide leads to improve cassava resistance to bacterial blight.

A cDNA-AFLP study revealed 78 differentially expressed transcripts (*ca.* 75% up regulated, 25% down regulated) 24h after cassava leaves were infiltrated with *Pst.* The fragments could be grouped according to their similarity to genes of known function (figure 6.8). Some of these genes have a potential role in the defence response, for example BCA23 is up-regulated and similar to a β 1-3 glucanase, a well characterised PR protein. Some other genes appear unrelated to defence, such as four up-regulated and three down-regulated TDFs that encode for proteins similar to RuBISCO. The potential roles of TDFs in defence will be discussed under four separate groupings: the hypersensitive response, signalling (local and systemic), production of antimicrobials and cell wall strengthening. The possible role of TDFs with similarity to ubiquitins will be discussed separately, as a clear role for ubiquitins in defence responses has yet to be elucidated.

6.4.1 Induction and maintenance of the hypersensitive response (HR)

The hypersensitive response (HR) is a pathogen-induced rapid and localised cell death (Heath, 2000). The HR is central to the induction of resistance in most cases of host and non-host resistance. Several requirements for HR induction have been identified including ion fluxes, protein synthesis, salicylic acid and an intact cytoskeleton (Heath, 2000). However most of these events occur long before the HR is visible (Grant and Mansfield, 1999). Any genes isolated in this study are likely to be involved in the progression of the HR and direct host cell killing.

Programmed cell death in animal cells is known to involve a variety of proteases (Cryns and Yuan, 1998). Proteases also play a role in plant programmed cell death (Beers *et al.*, 2000). Genes with similarity to three different classes of protease were induced in cassava leaves in response to *Pst.* BCA28, which is a longer version of BCA32, resembles a matrix metalloprotease from cucumber. BCA20 is similar to a proteasome precursor from *A.*

thaliana and BCA76 shows sequence homology to the ATPase subunit from the 26S proteasome of spinach. The metalloprotease BCA28 was upregulated in response to *Pst* but was not induced in response to infiltration with water. Metalloproteases use a water molecule activated by a divalent cation (normally zinc but it can be cobalt or manganese) to split a peptide bond (Beers *et al.,* 2000). Soybean metallo-endopeptidase (SMEP1) is localised in the apoplast and expressed highly during the late stages of leaf expansion (Graham *et al.*, 1991). SMEP1 has approximately 40% identity with animal matrix metalloproteases (MMPs) (McGeehan *et al.,* 1992). Animal MMPs include collagenases, gelatinases and stromalysins that are involved in degradation of the extracellular matrix (Beers *et al.*, 2000), required for morphogenesis, differentiation and wound healing (Werb, 1997). Extracellular MMPs also play a role in the control of programmed cell death in animals, for example weaning-induced apoptosis of mammary glands (Lefebvre *et al.,* 1992). SMEP1 is known to have a similar action to animal MMPs and may be involved in regulation of growth and development (McGeehan *et al.,* 1992). BCA28 may be involved in programmed cell death as expression was induced in leaves undergoing the HR. However, the extracellular localisation of SMEP1 suggests that BCA28 may be involved in attacking invading pathogens in the apoplast.

BCA20 and BCA76 are both similar to genes involved in the 26S proteasome. The 20S proteasome consists of four stacked rings of seven subunits forming a cylinder through which unfolded proteins enter and are digested to produce peptides of four to ten residues long (Coux *et al.*, 1996). The 26S proteasome is formed by the addition of a multisubunit cap. This cap makes the 26S proteasome ATP dependent and recognises ubiquitiated proteins that are then degraded by the 20S cylinder (Coux *et al.,* 1996). Several roles for proteasome dependent protein degradation have been shown, including cell cycle control, regulating transcription factors and regulating developmental and stress-induced programmed cell death (Ingvardsen and Veierskov, 2001). It is likely that the 26S proteasome is involved in programmed cell death in plants (Beers *et al.*, 2000). The up-regulation of proteasome subunits in cassava leaves undergoing the HR supports this hypothesis.

6.4.2 Local and systemic signalling

For a plant to be resistant to a pathogen it not only has to recognise that it is being attacked it also has to transmit the message to activate defence local and distant responses. Local signals need to be rapid in order to trigger the HR and kill the attacking pathogen before the pathogen can advance past the initial infection point (Blumwald *et al.,* 1998). Systemic

signals warn distal parts of the plant that it is under attack and increasing evidence points to methods of signalling between plants (Farmer, 2001).

Initial recognition events often lead to the initiation of the HR through a complex signalling pathway. This pathway(s) probably involves a combination of G proteins, changes in cytosolic calcium ion concentration and changes in protein phosphorylation (Blumwald *et al.*, 1998). Calcium is one of the most important messengers in plant signalling (Reddy, 2001) and is known to be crucial in the defence response (Grant *et al.*, 2000). Two up-regulated TDFs encode proteins with similarity to proteins involved in Ca^{2+} signalling. BCA1 resembles a Ca^{2+} transporting ATPase and BCA9 has similarity to calmodulin. Increases in cytosolic Ca^{2+} concentration is one of the first responses to elicitor binding in both suspension cells (Zimmermann *et al.*, 1997) and plants (Xu and Heath, 1998 and Grant *et al.*, 2000). The increase is only possible because Ca^{2+}-ATPase modulates the levels of intracellular calcium, so that the external [Ca^{2+}] is much higher than internal [Ca^{2+}]. Ca^{2+} channels are activated by both specific (Gelli *et al.*, 1997) and non-specific (Zimmermann *et al.*, 1997) elicitors, which cause an influx of Ca^{2+}. Specific fungal elicitors inhibit tomato Ca^{2+}-ATPase, possibly to prevent the establishment of the calcium gradient (Lam *et al.*, 1998). These responses are very rapid and it is unlikely that BCA1 up-regulation is involved in the initiation of the HR. However it is possible that the plant is sensitising its response mechanism by providing more Ca^{2+} translocating potential. The increase in [Ca^{2+}] is transferred to activate defence responses by calmodulin and Ca^{2+} dependent kinases/phospatases (Grant and Mansfield, 1999). Calmodulin binds calcium, which induces a conformation change and enables calmodulin to interact with target proteins (O'Neil and DeGrado, 1990). There are multiple calmodulin genes in plants that are either identical or differ in a few amino acid (Reddy, 2001). Different calmodulin isoforms are differentially expressed in response to different stimuli (Snedden and Fromm, 1998). In soybean cells treated with fungal elicitors specific calmodulin isoforms are induced (Heo *et al.*, 1999). Soybean contains five calmodulin isoforms, three of which are highly conserved with those from other plants and two of which show some divergence. The divergent isoforms are specifically induced by fungal elicitors and pathogens (Heo *et al.*, 1999). The different isoforms also show differential regulation of enzymes, including nitric oxide synthase that may be involved in triggering defence responses (Lee *et al.*, 2000). Differential expression of divergent isoforms of tobacco calmodulin in response to pathogen attack has also been reported (Heo *et al.*, 1999).

The systemic signalling that occurs in response to pathogen attack has been investigated in great detail in *A. thaliana*. Three signalling molecules appear to be involved, salicylic acid, jasmonic acid and ethylene (Thomma *et al.*, 2001). BCA59 and BCA66 have similarity to

ACC oxidase from *Prunus armeniaca.* ACC oxidase catalyses the formation of ethylene from the non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) (Bleecker and Kende, 2000). Ethylene is a gaseous hormone and is involved in a number of processes, in addition to defence against pathogens, including germination, senescence, fruit ripening and leaf abscission, (Stenpanova and Ecker, 2000). Ethylene-insensitive *A. thaliana* mutants have increased susceptibility to a number of, mainly necrotrophic, pathogens (Thomma *et al.*, 2001). Ethylene and jasmonate co-regulate a number of pathogenicity-related proteins (Thomma *et al.*, 1998). Because the biosynthesis of ethylene is up-regulated in response to pathogen attack it is unsurprising that ACC oxidase is up regulated in cassava leaves undergoing the HR.

BCA36 encodes a protein very similar to a WRKY transcription factor. Northern blot analysis shows that BCA36 is up-regulated in response to *Pst* but not in water inoculated controls. WRKY transcription factors are a superfamily with up to 100 representatives in the *A. thaliana* genome (Eulgem *et al.*, 2000). WRKY proteins bind the DNA sequence motif (T)(T)TCAG(C/T) known as the W box (Rushton *et al.,* 1995). The WRKY domain is a 60 amino acid region conserved between family members. It consists of the amino acid sequence WRKYGQK at the N terminus and a zinc-finger like motif (Rushton *et al.*, 1995). WRKY genes are expressed under many different physiological conditions and may control a number of biological functions. WRKY genes are up-regulated in response to viruses, bacteria, Oomycetes, fungal elicitors and various signalling molecules including salicylic acid (Eulgem *et al.,* 2000). The W box is found in the promoters of many plant genes induced in response to pathogen attack, including the WRKY genes themselves (Rushton and Sommsich, 1998). Transcription profiling experiments revealed a group of 45 genes that were co-regulated with PR gene expression. These genes contained on average four W boxes in the 1.1kb upstream of their start codons (Maleck *et al.*, 2000). In contrast, genes differentially expressed but not clustering with PR gene expression had no more than two W boxes 1.1kb upstream of the start codon (Maleck *et al.,* 2000). This indicates that antimicrobial proteins are up regulated in response to the action of WRKY proteins. WRKY proteins appear to be a large family of plant specific transcription factors that help to co-ordinate responses to stresses. The discovery of the first cassava WRKY protein may help to understand the defence responses of cassava.

6.4.3 Production of antimicrobials

The induction of phytoalexin biosynthesis is a well-characterised response to pathogen attack (Hammerschmidt, 1999). However the genes involved in phytoalexin biosynthesis and regulation are poorly understood (Dixon, 2001).

Four up-regulated and one down-regulated TDFs are similar to genes involved in secondary metabolism and may be involved in phytoalexin production. BCA34 is similar to 3-deoxyarabinoheprulosonate 7- phosphate synthase (DAHP synthase). DAHP synthase is an enzyme in the shikimate pathway and is the first committed enzyme of aromatic amino acid biosynthesis. Aromatic amino acids are used both in proteins and for the production of an array of secondary metabolites, notably phenylalanine and tyrosine. *A. thaliana* has two copies of DAHP synthase and one is induced in response to wounding and to attack by avirulent pathogens (Keith *et al.,* 1991). Many genes of aromatic amino acid biosynthesis are duplicated in plants, which may indicate separate pathways for amino acids for proteins and amino acids for secondary metabolites (Keith *et al.*, 1991). Whilst no other DAHP synthase from cassava has been cloned it is likely that the one isolated in this study is homologous to the inducible version in *A. thaliana.* Indeed BCA34 is more similar to *A. thaliana* DAHP1 (inducible) than to DAHP2 (constitutive). BCA41 is similar to S-adenosyl-l-homocysteine hydrolase (SAH hydrolase), a regulatory enzyme in methyltransferase reactions (Mitsui *et al.*, 1997). Methylation is an important reaction in the synthesis of antimicrobial compounds. Methyl groups are donated by S-adenosyl-L-methionine (SAM) and the resulting S-adenosyl-L-homocysteine (SAH) is hydrolysed by SAH hydrolase to L-homocysteine and adenosine. SAH is a potent inhibitor of SAM dependent methyltransferases and SAH hydrolase is required to maintain methylation of stress-induced compounds (Weretilnky *et al.*, 2001). SAH hydrolase activity has been shown to double following the addition of fungal elicitors to alfalfa cells (Edwards, 1996). BCA5 and BCA51 are both similar to enzymes involved in the biosynthesis of anthocyanins. BCA51 is similar to dihydroflavanol reductase (DHF reductase). DHF reductase catalyses the conversion of dihydroflavanol to flavan-3, 4-diol (Dixon and Paiva, 1995). The down-regulated BCA5 is similar to an UDP-glucose flavanoid 3-O-glucosyl transferase (UFG transferase). UFG transferase catalyses the conversion of anthocyanidin to anthocyanin (Dixon and Paiva, 1995). Northern blots probed with the BCA5 TDF showed a down-regulation of expression after infiltration with both water and *Pst.* This down-regulation was more marked with *Pst* infiltration. The BCA51 TDF appeared to show up-regulation in response to both water and *Pst.* If these results are confirmed and protein levels follow mRNA levels one would expect an accumulation of anthocyanidin in response to pathogen attack. Whilst anthocyanins are not known phytoalexins, stress-induced accumulation of these compounds has been observed. Anthocyanins increase in response to high visible light, possibly due to photoprotective properties (Beggs *et al.,* 1987). Anthocyanins also increase following cold and nutritional stresses, but the function in these responses is unclear (Christie *et al.,* 1994).

BCA45 and BCA60 are similar to a cytochrome P450s. P450s are heme- containing mixed function oxidases found in all kingdoms (Feldmann, 2001). In plants, P450s are involved in the phenyl-propanoid, terpenoid and alkaloid biosynthetic pathways. They are known to be involved in phytoalexin biosynthesis and to be induced in response to pathogen attack (Whitbred and Schuler, 2000). Two *A. thaliana* P450 enzymes were recently found to contain several WRKY *cis*-elements, indicating that they are under the control of pathogen-inducible transcription factors (Maleck *et al.*, 2000). A P450 is required for the production of the phytoalexin camalexin in *A. thaliana* (Zhou *et al.*, 1999) and four P450s are required for the biosynthesis of the maize phytoanticipin DIMBOA (Frey *et al.*, 1997). Another defence-related compound produced by P450s is the cyanogenic glycoside dhurrin from sorghum (Kahn *et al.*, 1997). Two P450s are induced in pea in response to pathogen attack and may be involved in the biosynthesis of phytoalexins (Whitbred and Schuler, 2000). Other possible defence-related reactions catalysed by P450s include the production of phytohormones (Bak *et al.*, 2001). However P450s are a large group of enzymes with an estimated 273 genes in *A. thaliana* catalysing a number of reactions (Feldmann, 2001). It is impossible to conclude that the P450s isolated in this study are involved in the defence response without further study.

6.4.4 Cell wall strengthening

Lignin deposition is a well-known plant response to pathogens (Ride, 1983). The biosynthetic enzymes of lignin are transcriptionally regulated both by developmental and environmental cues (Whetten and Sederoff, 1995). Lignin biosynthesis is divided into two stages, the production of lignin monomers (monolignols) from phenylalanine and the polymerisation of these monomers to give lignin (Whetten and Sederoff, 1995). The initial enzyme in lignin, and all phenylpropanoid, biosynthesis is phenylalanine ammonia lyase (PAL), which is induced by pathogen attack (Dixion and Paiva, 1995). PAL transcripts were induced in cassava leaves infiltrated with *Xam, Xcc* (Pereria *et al.*, 1999) and *Pst* (this study).

BCA44 is similar to cinnamoyl coenzyme A reductase (CCR). CCR is speculated to be a key regulatory enzyme in lignin biosynthesis (Goffner *et al.*, 1994). CCR catalyses the reduction of hydroxylcinnamoyl-CoA thioesters to the corresponding aldehydes and is the first committed step in the production of monolignols from phenylpropanoid metabolism (Whetten and Sederoff, 1995). *A. thaliana* has two forms of CCR, *AtCCR1* is expressed constitutively whereas *AtCCR2* is induced in response to incompatible *X. c.* pv. *campestris* (Lauvergeat *et al.*, 2001). It is possible that a similar system is present in cassava.

The polymerisation of monolignols into lignin is an enzymatic oxidative process that is not well understood (Dean and Eriksson, 1994). Laccase, an oxygen dependent oxidase, or peroxidase, an H_2O_2 dependent oxidase, may be the enzyme responsible (Dean and Eriksson, 1994). Two differentially expressed TDFs are similar to peroxidases (BCA70 and BCA74). Up-regulation of peroxidase genes in response to pathogen elicitors is well documented (e.g. Smith *et al.,* 1991, Rasmussen *et al.*, 1995 and Kawalleck *et al.*, 1995). It is unlikely that the peroxidase arising from this up-regulation is involved in the pathogen-induced oxidative burst, because of rapidity of the initial burst (Lamb and Dixon, 1997). It is feasible that the two TDFs with similarity to peroxidases are either involved in lignification or in the oxidative cross-linking of cell walls.

Peroxidase catalyses the reaction H_2O_2 + OH-R-OH → $2H_2O$ + O=R=O. If the R group is a monolignol it is converted from a diphenol to a diquinone which leads to lignin polymerisation (Lamb and Dixon, 1997). Peroxidase can also catalyse the production of the reactive oxygen species O_2^- and H_2O_2. This reaction may be involved in the generation of H_2O_2 for lignin biosynthesis (Halliwell, 1978). Peroxidase activity has been monitored in cassava plants responding to xanthomonads (Pereira *et al.*, 2000). Cell wall bound peroxidase activity was higher in MCol22 plants inoculated with *X. cassavae* than in plants inoculated with *Xam* (Pereira *et al.*, 2000). MCol22 is resistant to *X. cassavae* but susceptible to *Xam* and the authors suggested that the peroxidase activity may be involved in this resistance (Pereira *et al.*, 2000).

BCA26 is similar to an oxalate oxidase from apple. Oxalate oxidase transcripts are increased in both wheat (Hurkman and Tanaka, 1996) and barley (Zhang *et al.,* 1995) in response to powdery mildew. Transcription increases are probably too slow to instigate the oxidative burst but may be involved in oxidative cross-linking of cell walls (Lamb and Dixon, 1997). However oxalate oxidase belongs to a large family of proteins (the germin and germin-like proteins) with many functions (Bernier and Berna, 2001) and a role in defence is not definite.

6.4.5 Ubiquitin

Ubiquitin is a small, reusable polypeptide tag. Attachment of ubiquitin to proteins targets them for degradation by the proteasome (Ingvardsen and Veierskov, 2001). In all, five TDFs had similarity to ubiquitins. One (BCA12) had increased expression on infection whilst the others (BCA21, BCA30, BCA48 and BCA52) showed down-regulation. The five TDFs were not identical and each had highest similarity to a different ubiquitin. *A. thaliana* has 82 ubiquitin-like proteins in its genome, compared with eight in yeast and 32 in humans (Bachmair *et al.,* 2001). The role of these different proteins has yet to be

resolved (Bachmair *et al.,* 2001). Increased ubiquitin expression has been reported in potato leaves infected with *Phytophthora infestans* (Basso *et al.* 1996), soybean in response to *Pseudomonas syringae* pv. *glycinea* (Seehaus and Tenhaken, 1998) and soybean cell cultures under pathogen-induced oxidative stress (Levine *et al* 1994). Microarray experiments showed down-regulation of an *A. thaliana* ubiquitin under a number of conditions, including chemically induced systemic acquired resistance (SAR) and in response to both compatible and incompatible *Peronospora parasitica* isolates (Maleck *et al.*, 2000). However no clear pattern of expression was seen; pathogen-induced SAR and a number of SAR related mutants showed no change in expression compared to untreated plants (Maleck *et al.*, 2000). When a mutant form of ubiquitin was over-expressed in tobacco plants spontaneous HR lesions resulted (Becker *et al.,* 1993). This suggests a role for ubiquitin in the regulation of cell death. Effector proteins from some phytopathogenic bacteria are suggested to interfere with ubiquitination, based on homology to the YopJ protein from *Yersinia* spp. that interferes with ubiquitination and protein degradation in animal cells (Lahaye and Bonas 2001). However the role of ubiquitin in plant defence is far from being understood.

Chapter 7

General Discussion

This thesis has investigated cassava bacterial blight from both the plant and pathogen sides of the disease. This "holistic" approach has revealed some of the potential mechanisms of cassava resistance to bacterial diseases and pathogenicity and virulence determinants of *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). Initial experiments to elucidate levels of resistance to CBB of the parents of the molecular genetic map of cassava (MNga2 and CM2177-2) showed no significant differences in levels of resistance, suggesting that the molecular genetic map will have limited use in discovering genes involved in resistance to bacterial blight (chapter 3). Because resistance to CBB is polygenic it is difficult to dissect using conventional genetic techniques, a task made almost impossible when the two cultivars under investigation have a similar resistance phenotype. Previous attempts to use MNga2 and CM2177-2 to isolate quantitive trait loci (QTLs) for resistance to CBB have met with limited success, with only one of 20 potential resistance loci being conserved between field and glasshouse trials (Jorge *et al.,* 2000; Jorge *et al.,* 2001). For any sensible studies leading to the isolation of resistance determinants model cassava cultivars for reactions to *Xam* are needed, and it is possible that a screen of wild *Maniot* spp. using a variety of inoculation methods will isolate cultivars with significant levels of resistance to *Xam*, which could be used to determine resistance mechanisms.

The strain of *Xam* (I56) used to assess levels of cultivar resistance was also used to determine potential virulence or pathogenicity factors of *Xam.* Extracellular polysaccharide (EPS) from *Xam* appeared to be synthesised from the same gene products found in *X. campestris* pv. *campestris*, *Xylella fastidiosa, X. axonopodis* pv. *citri* and *X. oryzae* pv. *oryzae* (chapter 4) (Katzen *et al.*, 1998; da Silva *et al.*, 2001; da Silva *et al.*, 2002; Dharmapuri and Sonti, 1999). The high conservation of sequences from these pathogens with highly divergent host ranges indicates that EPS production is an ancient phenomenon and is not involved in determining host range. EPS$^-$ strains of *Xam* I56 were constructed with defined, marker-free deletions in *gumD* and severely attenuated virulence (chapter 4). However, EPS$^-$ strains multiplied at the infection point, indicating that EPS may not be required for initial survival in the host, but is essential for spread and dissemination. Further study is needed to elucidate the exact roles of EPS from *Xam* and how it contributes to pathogen fitness. Possible roles suggested by this study include resistance to antimicrobials, suppression of host defences and spread and dissemination. It is possible that EPS-degrading enzymes could be used to increase resistance of transgenic cassava to CBB. All strains of *Xam* appear to produce EPS therefore these transgenics could provide relatively durable resistance. Because of recent adverse publicity concerning transgenic

crops (especially those targeted at developing countries) the possibility of cassava plants having EPS-degrading enzymes must be investigated for conventional breeding techniques. Cassava latex appears to be a rich source of carbohydrate-degrading enzymes (chapter 5) and this may be a good starting material.

Studies into the role of pectate lyase (Pel) as a potential pathogenicity or virulence factor showed that production *in vitro* was not universal in all strains of *Xam* and the *pelA* gene did not appear to be present in all strains tested (chapter 4). The function of pectolytic enzymes in xanthomonads is not clear, but they do not appear to be essential for either pathogenicity or virulence (Liao *et al.*, 1996) and production of pectolytic enzymes is not universal in xanthomonads (Dye, 1968). Most xanthomonads appear to have biotrophic tendencies and probably gain energy from the plant by subtler means than direct attack of the cell wall, pectolytic degradation of cell walls is usually associated with host cell death (Cooper, 1983). Conversely attacking the plant cell wall could release potential oligosaccharide elicitors of defence responses. However xanthomonads do survive outside the host and pectolytic enzymes may be involved in saprophytic life. Elucidating the function of Pel in *Xam* should involve the construction of a strain of *Xam* with a defined deletion in *pelA* then assessment of virulence, pathogenicity and saprophytic survival.

Whilst no total resistance to CBB has been reported, cultivars with extreme differences in reaction to *Xam* do exist (K. Wydra, personal communications). The field resistance levels of two such cultivars, BEN, which is very susceptible to CBB and MNga2, which is relatively resistant, may in part be due to differences in stomatal density on the adaxial leaf surface (chapter 5). However no correlation was found between stomatal density and resistance to CBB in four glasshouse grown cultivars (chapter 5). Stomates are an obvious portal of entry for phytopathogens, and their structure, distribution and density have been investigated as potential disease resistance determinants in a number of species. Only in a few cases have correlations between stomatal characteristics and disease resistance been shown (Royle, 1975). Whether these correlations are entirely due to differences in stomatal characteristics or other effects has not been established. For stomatal characteristics to be considered a resistance determinant, a number of basic facts need to be established. 1) Does the pathogen enter through the stomates? *Xam* has been reported to enter cassava leaves through the stomates of *in vitro* grown cassava (Verdier *et al.*, 1990) but no hard evidence for entry through the stomates of fully developed cassava leaves has been reported. The author has constructed a strain of *Xam* I56 that expresses GUS *in planta,* but entry of this strain into the stomates of cassava has yet to be shown despite several attempts. 2) Does significant stomatal polymorphism exist between cultivars? This study and others (El-Shakawy *et al.*, 1984) indicate that adaxial stomatal density does vary with

cassava cultivar, although, based on evidence from other *Xathomonas* diseases, differences in structure and distribution of stomates could be important in determining resistance, this aspect has not been investigated sufficiently in cassava. 3) Do differences in stomatal characteristics absolutely correlate with disease susceptibility? In this study a correlation is apparent with field grown cultivars, though only with two cultivars. However glasshouse grown cultivars showed no correlation with stomatal distribution and disease resistance. More investigation of these points is needed before any firm conclusions on the role of stomates in resistance to CBB can be drawn. Indeed, some of the techniques developed during this work have been transferred to African researchers at IITA, Benin. However it must be remembered that one of the main causes of CBB spread is through infected tools (Lozano, 1986) and portals of entry other than stomates (for example wounds and insect vectors) exist, which would not be prevented by fewer adaxial stomates. Cassava is not the ideal species to address the broader questions concerning function of stomatal characteristics in disease resistance. Recently several *A. thaliana* mutants have been identified with altered stomatal density, morphology and distribution (Brownlee, 2001). The susceptibility of these mutants to a stomate-infecting pathogen may help to resolve the role of stomates in disease resistance.

In addition to stomatal characteristics two other potential preformed resistance mechanisms of cassava to CBB were investigated in this study; the components of cassava latex and the partial isolation of a possible phytoanticipin. The function of latex is assumed to be as a defensive barrier; sealing wounds, clogging the mouthparts of insects and having antifungal, antibacterial and antiviral properties. Cassava latex contained enzyme activities from a number of pathogen response (PR) proteins and a small EST library of cassava latex revealed that approximately one-third of latex transcripts encoded proteins involved in defence against biotic or abiotic stress (chapter 5). These proteins ranged from "classic" PR proteins (e.g. glucanase, chitinase) to a recently isolated gene involved in defence against viruses *via* gene silencing (*argonaute*), indicating that at least one role of cassava latex is in defence. Studies of latex in other species have revealed the presence of antimicrobials, both proteins and low molecular weight compounds (e.g. Martin, 1991; Sessa *et al.*, 2001). To investigate the properties of cassava latex in terms of understanding its function a systematic approach needs to be taken. Sequencing more ESTs coupled with analysis of the proteins and metabolites present in cassava latex would reveal if it has a function solely as a defensive mechanism or if other roles (such as a storage system) are apparent. In addition, because of the unique physical properties of latex (notably rapid coagulation), an effective assay for antimicrobial activity was not developed during this study, something that must be addressed in the future. In terms of resistance to CBB, latex

may provide a useful defensive barrier against insect vector borne *Xam*. The antimicrobial properties of latex may vary between cassava cultivars, possibly accounting for differing resistance levels to *Xam*.

Partial isolation of a phytoanticipin(s) in the ethyl acetate soluble extract of cassava leaves with activity against *Xam* (chapter 5) indicates that cassava leaves have the ability to inhibit or kill *Xam*. This begs the question of why is *Xam* not inhibited by the potentially toxic environment? Further work includes identifying the compound(s), determine if the compound(s) are compartmentalised and determine if *Xam* has the ability to detoxify or avoid contact with the compound(s) *in planta*. It is possible that the activity observed was an artefact of the extraction process; chloroform (with a similar polarity to ethyl acetate) failed to extract antimicrobial compounds from unchallenged cassava leaves. Whilst a large number of low molecular weight antimicrobial compounds have been isolated from plants, only a small percentage appear to have a definite role in defence (Dixon, 2001) and it is unlikely that the antibacterial compound(s) in this study has a pivotal role in determining resistance to CBB. However they may be a component of the complex, multicompent mechanisms of cassava defence and resistance to CBB could be improved either by engineering cassava plants to express the antimicrobial activity on infection with *Xam* or by screening for cultivars (or wild relatives) with higher levels of antimicrobial activity.

Because of the poor expression of resistance to *Xam* a more reliable inducer of cassava defences was found in the tomato pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*). cDNA-AFLP revealed that *ca.* 1% of the cassava transcriptome is differentially regulated in response to *Pst* (chapter 6). This is similar to the level of differential regulation seen with tomato infected with *Cladosporium fulvum* (Durrant *et al.*, 2000) and *A. thaliana* infected with a number of pathogens (Maleck *et al.*, 2000). A number of novel cassava genes have been isolated that were induced in response to *Pst* including a metalloprotease, a WRKY transcription factor and a calmodulin-like protein (chapter 6). The kinetics of BCA gene expression in cassava leaves infected with *Xam* needs to be ascertained to see if they are involved in resistance to CBB or just a component of non-host resistance. It is likely that, as in *A. thaliana* (Maleck *et al.*, 2000), non-host and host resistance responses of cassava are similar, but the timing of responses may differ. If possible, further transcriptome characterisation would utilise microarrays, providing a cassava chip of sufficient coverage and quality became available. The genes isolated to date need to be characterised in greater detail, especially those which have not previously been described in response to plant pathogens (such as the metalloprotease, which could be involved either in the HR or as a new PR protein) or are similar to genes of no known function. The final

utility of such information would be in identifying markers for resistance to CBB that could be used to breed improved lines.

Considering the considerable suffering which could be inflicted by an outbreak of CBB in the developing world, CBB has not received the research attention it deserves. This study indicates some of the areas of CBB worthy of future research; pathogenicity determinants of *Xam* as potential targets for control, the characteristics of preformed and induced defences as features for a screen or to manipulate genetically. However in order to develop more information about CBB, model cultivars of cassava, with distinct and reproducible differences in resistance phenotype, and standard strains of *Xam* need to be adopted for all CBB researchers.

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San Diego supercomputing centre biology workbench; http://workbench.sdsc.edu/

National Centre for Biological Information (Genbank) http://www.ncbi.nlm.nih.gov/

Human genome mapping project; http://www.hgmp.mrc.ac.uk

Appendices

Appendix 1 Culture Media

Unless stated media components are from Oxoid (Basingstoke, UK), general chemicals from Merck Eurolabs and antibiotics from Melford Laboratories.

NYGA (Nutrient Yeast Glycerol Agar) Medium

For growth of xanthomonads and phytopathogenic pseudomonads (Turner *et al.*, 1984)

15g bacteriological peptone

3g yeast extract

20g glycerol

14g technical agar

Make up to 1l with distilled H_2O and sterilise by autoclaving at 121°C for 20 min. For NYGB (Nutrient Yeast Glycerol Broth) omit agar from above.

CTA (Cefazolin Trehalose Agar) Medium

A semi-selective medium for the growth of *Xam* (Fessehaie *et al*, 1999). EPS minus strains do not grow as well on this medium as wt stains.

3g K_2PO_4

1g NaH_2PO_4

0.3g $MgSO_4 \cdot 7H_2O$

1g NH_4Cl

9g D(+) trehalose

1g glucose

1g yeast extract

14g agar

Make up to 1l with deionised water and adjust pH to 7.1. Sterilise by autoclaving at 121°C for 20 min. Cool to *ca.* 55°C and add 2.5ml filter sterile 10mg ml^{-1} cefazolin, 100µl filter sterile 12mg ml^{-1} lincomycin, 1ml filter sterile 2.5mg ml^{-1} phosphomycin and 1ml filter sterile 250mg ml^{-1} cycloheximide (Merck Eurolabs).

Minimal Medium

For the growth of *Xam* and induction of cell wall degrading enzyme production (Deshappriya, 1992).

0.5g $NH_4H_2PO_4$

0.2g $MgSO_4 \cdot 7H_2O$

0.5g K_2HPO_4

2.0g NaCl

Make up to 1l with deionised H_2O and sterilise by autoclaving. Cool to room temperature before adding 1ml filter sterile 1% (w/v) $CaCl_2 \cdot 2H_2O$ and 1ml filter sterile 10mg ml^{-1} L-methionine. A carbon source (e.g. 1% (w/v) cassava cell walls) is then added.

LB (Luria-Bertani) Medium

For routine growth of *E. coli* strains

10g bacteriological tryptone

5g yeast extract

10g NaCl

15g Agar

Make up to 1l with distilled H_2O and sterilise by autoclaving at 121°C for 30 minutes. Agar should be cooled to *ca.* 55°C prior to the addition of any antibiotics or histochemical reagents. For liquid medium omit agar from above. For LB when working with λ phage add 2g maltose and 2.46g $MgSO_4 \cdot 7H_2O$ prior to autoclaving

SOC medium

For growth of *E. coli* for making competent cells

20g bacteriological tryptone

3g yeast extract

10ml 1M NaCl

2.5ml 1M KCl

Make up to 980ml with distilled H_2O and sterilise by autoclaving at 121°C for 30 minutes. Cool to room temperature and add 10ml filter sterile 2M Mg^{2+} (1M $MgCl_2$, 1M $MgSO_4$) and 10ml filter sterile 2M glucose. For SOB omit glucose.

NZY agar / top agar

For plating out phage

5g NaCl

2g $MgSO_4 \cdot 7H_2O$

5g yeast extract

10g casein hydrolysate

15g agar (7g agar for top agar)

Make up to 1l with distilled H_2O and sterilise by autoclaving at 121°C for 30 minutes.

Appendix 2 Buffers

Sodium Phosphate Buffer (0.1M)

For the correct pH add *x* ml 0.2M $Na_2HPO_4 \cdot 2H_2O$ and *y* ml 0.2M $Na_2H_2PO_4 \cdot H_2O$ and make solution up to 100ml.

pH	*x* (0.2M $Na_2HPO_4 \cdot 2H_2O$)	*y* (0.2M $Na_2H_2PO_4 \cdot H_2O$)
5.8	4	46
6.0	6.15	43.85
6.2	9.25	40.75
6.4	13.25	36.75
6.6	18.75	31.25
6.8	24.5	20.5
7.0	30.5	19.5
7.2	36.0	14.0
7.4	40.5	9.5
7.6	43.5	6.5
7.8	45.75	4.25
8.0	47.35	2.65

PBS (Phosphate Buffered Saline)

8g NaCl

0.2g KCl

1.44g Na_2HPO_4

0.24g KH_2PO_4

Dissolve in 800ml deionised H_2O and adjust pH to 7.4 with HCl. Make up to 1l and sterilise either by autoclaving or filtration.

Appendix 3 PCR primers

All primers are shown 5' to 3'

Amplification of 569bp of *gumD* from *Xam*

gumD5' TTGCTGAGTGAGCTGGTGGT

gumD3' GTTGATCACCGGCACGCTGC

Amplification of 430bp of *pelA* from *Xam*

pelA3' GAACGGGTAGACCTTATTGCTGG

pelA5' GATCATCCGCAACATCACCATCG

Amplified Fragment Length Polymorphism (AFLP)

AFLP primers are as detailed in Durrant *et al.*, 2000.

***Apo* I adapters**

WD20 CTCGTAGACTGCGTACC

WD50 AATTGGTACGCAGTCTAC

***Mse* I adapters**

WD47 GACGATGAGTCCTGAG

WD48 TACTCAGGACTCAT

Primers for preamplification of template

WD16 CTCGTAGACTGCGTACAAATT (*Apo* I)

WD19 GACGATGAGTCCTGAGTAA (*Mse* I)

***Mse* I primers with two selective bases**

WD31- WD46 GAT GAGTCCTGAGTAANN

Where N represents any base, giving a total of 16 different primers

***Apo* I primers with one selective base**

Eco RI +A – *Eco* RI +T GACTGGGTACCAATTCN

WD6- WD9 GACTGGGTACCAATTTN

Where N represents any base, giving a total of 8 different primers

Primers used in RT-PCR of TDFs

The predicted function of the TDF (or the gene of highest similarity) is shown.

BCA2 Protein kinase

BCA2 5' CCTGTTTACGATGTTGGGAT

BCA2 3' CAGCGAGTTATGCAAGCTCG

BCA3 Drosphila transcriptional regulator

BCA3 5' CAAGCAGAAGCATACGCCTG

BCA3 3' ACACCATATAGAAGCAGCAA

BCA5 Flavonol 3-O-gluxosyltransferase 6

BCA5 5' TGTGGAGCCCATCCAACTAC

BCA5 3' CCAGTGGGACCCATCTTGAA

BCA9 Calmodulin like protein

BCA9 5' GATGGAGACATGTTCTTAGC

BCA9 3' GAGGTGGCAAGAGCACCATT

BCA14 Malate dehydrogenase

BCA14 5' GAAGATTTTGCTAACCACAA

BCA14 3' CAATGCTCATGAGGCCAAGG

BCA20 Multicatalytic endopeptidase complex

BCA20 5' CATCCCCTCAATTAGACCTG

BCA20 3' GGATCTCAAGGCTCCTCACT

BCA22 Arabidopsis predicted protein of unknown function

BCA22 5' AACAACATTCTCGTCCAAGC

BCA22 3' ACCTTGGATTGATACTATGC

BCA23 Glucanase

BCA23 5' GACCTCAAGGTATTTACACG

BCA23 3' CCTGTTTACGATGTTGGGAT

Appendix 4: Statistical analysis of EPS$^-$ bacterial infections

Minitab for Windows was used for all statistical analysis. The outputs from some Minitab calculations are presented here.

Bacterial Numbers

Because bacterial numbers were determined from less than seven plants the Mann-Whitney U test was used to test for significance. Experiments using bacterial numbers obtained at the initial site of infection (figure 4.12) were compared at 14dpi (where the greatest difference between the WT and EPS$^-$ numbers is expected

Mann-Whitney Confidence Interval and Test for Number of EPS$^-$ and WT Bacteria at Site of Initial Infection 14 dpi

```
WT    N=6          Median =    143333333
EPS-  N=6          Median =    104166667
Point estimate for ETA1-ETA2 is      39166669
95.5 Percent CI for ETA1-ETA2 is (-56666665,308333297)
W=45.0
Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.3785

Cannot reject at alpha = 0.05
```

This analysis shows that there is no significant difference at the 95% confidence interval between the number of WT bacteria at the initial infection point and the number of EPS$^-$ bacteria at the initial infection point 14dpi.

Mann-Whitney Confidence Interval and Test for Number of EPS$^-$ and WT Bacteria 1cm Away from the Site of Initial Infection 32dpi

```
WT    N=5          Median   =   2083333
EPS-  N=6          Median   =        92
Point estimate for ETA1-ETA2 is 2083241
96.4 Percent CI for ETA1-ETA2 is (1066600,4016551)
W = 45.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0081
The test is significant at 0.0080 (adjusted for ties)
```

This analysis shows that there are significantly (P=0.008) more WT bacteria present 1cm away from the initial site than EPS$^-$ bacteria 1cm away from the initial infection site at 32dpi.

Mann-Whitney Confidence Interval and Test for Number of EPS$^-$ and WT Bacteria in a Whole Lobe 32dpi

```
WT      N =    7          Median =  9833333333
EPS-    N =    4          Median =   120833333
Point estimate for ETA1-ETA2 is  9712500257
95.3 Percent CI for ETA1-ETA2 is (4743996135,22080005363)
W = 56.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0107
```

This analysis shows that there are significantly (P=0.01) more WT bacteria present in a lobe than EPS$^-$ bacteria present in a whole lobe at 32dpi.

Appendix 5: Statistical Analysis of Resistance of Three Cassava Cultivars

Minitab for Windows was used for all statistical analysis. Because of the large number of samples it was possible to use a t-test to determine levels of significance. In order to use the t-test data must be normal (Gaussian) so the normality of the data was tested using the Kolmogorov - Smimov normality test. Sets of data were then compared to each other using a two-sample t-test assuming unequal variance (normal data) or a Mann-Whitney U test (non-normal data). Selected data sets were compared with both tests to ensure analysis was correct. An example of the Minitab output is shown below comparing the symptom index (SI) of MCol22 27dpi with the SI of MNga2 27dpi both with a two-sample t-test and a Mann-Whitney U test. Other analysis is summarised in the table following.

Mann-Whitney Confidence Interval and Test comparing SI on MCol22 and MNga2 27dpi following leaf infiltration with 1x10^6 cfu ml^{-1} *Xam* I56

```
27daysMC    N=26        Median =    3.5000
27daysMN    N=32        Median =    3.0000
Point estimate for ETA1-ETA2 is     0.5000
95.0  Percent CI for ETA1-ETA2 is (0.0001,0.4998)
W = 944.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0009
The test is significant at 0.0007 (adjusted for ties)
```

Two Sample T-Test and Confidence Interval comparing SI on MCol22 and MNga2 27dpi following leaf infiltration with 1x10^6 cfu ml^{-1} *Xam* I56

```
Two sample T for 27daysMC vs 27daysMN
             N     Mean       StDev    SE Mean
27daysMC    26    3.596       0.600       0.12
27daysMN    32    3.141       0.675       0.12
95% CI for mu 27daysMC - mu 27daysMN: ( 0.12, 0.79)
T-Test mu 27daysMC = mu 27daysMN (vs not =): T = 2.72 P = 0.0088 DF = 55
```

Both these analysis indicate a significant difference between the SI on MCol22 and the SI on MNga2 27dpi following leaf infiltration with 1x10^6 cfu ml^{-1} *Xam* I56. However the levels of significance are much higher using the Mann-Whitney U test (P=0.0007)

compared with the t-test (P=0.0088). This is because the Mann-Whitney test is based on the median and the t-test based on the mean and the tests were highly variable.

Leaf infiltration with $1x10^6$ cfu ml^{-1} *Xam*

Cultivar	mean SI ± stdev	p vs. CM2177-2	p vs. MCol22	p vs. MNga2
CM2177-2 8 dpi	0.177±0.304		**0.0000**	0.35
MCol22 8 dpi	0.827±0.314	**0.0000**		**0.0000**
MNga2 8 dpi	0.250±0.311	0.35	**0.0000**	
CM2177-2 15 dpi	1.016±0.491		**0.0000**	0.055
MCol22 15 dpi	2.058±0.668	**0.0000**		**0.0000**
MNga2 15 dpi	1.250±0.453	0.055	**0.0000**	
CM2177-2 27 dpi	2.48±1.24		**0.0001**	**0.014**
MCol22 27 dpi	3.60±0.60	**0.0001**		**0.0088**
MNga2 27 dpi	3.14±0.67	**0.014**	**0.0088**	

All data sets were normal as determined by the Kolmogorov - Smimov normality test and a two-sample t-test was conducted on the data assuming unequal variance. The table shows cultivar on the left-hand column, followed by the mean SI and standard deviation and the p value when compared to the data sets from other cultivars at the same time point. Where a very low p value was obtained the value 0.0000 is shown (the output from Minitab). P values less than 0.05 (shown in **bold**) indicate a significant difference between the two cultivars at this time point.

Leaf infiltration with $1x10^4$ cfu ml^{-1} *Xam*

Cultivar	mean SI ± stdev	p vs. CM2177-2	p vs. MCol22	p vs. MNga2
CM2177-2 15 dpi	0.306±0.358		**0.0000**	0.14
MCol22 15 dpi	1.077±0.366	**0.0000**		**0.0000**
MNga2 15 dpi	0.438±0.330	0.14	**0.0000**	
CM2177-2 22 dpi	1.000±0.592		**0.0000**	0.17
MCol22 22 dpi	1.692±0.549	**0.0018**		**0.0000**
MNga2 22 dpi	1.203±0.580	0.17	**0.0018**	
CM2177-2 27 dpi	1.250±0.861		**0.0068**	0.35
MCol22 27 dpi	1.920±0.909	**0.0068**		0.054
MNga2 27 dpi	1.453±0.855	0.35	0.054	

All data sets were normal as determined by the Kolmogorov - Smimov normality test and a two-sample t-test was conducted on the data assuming unequal variance. The following

table shows cultivar on the left-hand column, followed by the mean SI and standard deviation and the p value when compared to the data sets from other cultivars at the same time point. Where a very low p value was obtained the value 0.0000 is shown (the output from Minitab). P values less than 0.05 (shown in **bold**) indicate a significant difference between the two cultivars at this time point.

Petiole stab with $1x10^6$ cfu ml^{-1} *Xam*

Cultivar	mean SI ± stdev	median	p vs. CM2177-2	p vs. MCol22	p vs. MNga2
CM2177-2 15 dpi	0.161±0.299	0.000		**0.0017**	0.3491
MCol22 15 dpi	0.540±0.498	0.500	**0.0017**		**0.0317**
MNga2 15 dpi	0.266±0.401	0.000	0.3491	**0.0317**	
CM2177-2 22 dpi	1.096±1.457	1.000		**0.0365**	0.7273
MCol22 22 dpi	1.804±1.629	1.000	**0.0365**		0.0648
MNga2 22 dpi	1.015±1.066	1.000	0.7273	0.0648	
CM2177-2 27 dpi	2.484±2.161	2.250		0.7812	0.4525
MCol22 27 dpi	2.576±1.653	2.000	0.7812		0.2163
MNga2 27 dpi	2.109±1.702	1.750	0.4525	0.2163	

The standard deviations are very high for all data sets and some were found to be non-normal as determined by the Kolmogorov - Smimov normality test. This is because to a large extent the petiole stab is an "all or nothing" inoculation technique, where leaves either wilt or remain healthy. The Mann-Whitney U test was used to test levels of significance between data sets. The following table shows cultivar on the left-hand column, followed by the mean SI and standard deviation, the median (which is used in the Mann-Whitney test) and the p value when compared to the data sets from other cultivars at the same time point. Where a very low p value was obtained the value 0.0000 is shown (the output from Minitab). P values less than 0.05 (shown in **bold**) indicate a significant difference between the two cultivars at this time point.

Appendix 6:- Similarities of Latex ESTs to genes of known function.

Sequences were compared to the database using BlastX. The gene with highest similarity to the EST is listed

Genes speculated to be involved in stress tolerance

Similarity	Number of ESTs in library
Acidic Endochitinase from poplar (U01661)	16
Dehydration-induced protein RD22 from *A. thaliana* (D10703)	4
β1,3-glucanase from Rubber tree (U22147)	3
Nodulin from Alfalfa (L18899)	2
Trypsin Inhibitor from Cucumber (S12897)	2
Basic chitinase from *Arabis holboellii* (AF135130)	1
Desiccation-related protein from *A. thaliana* (AC051631)	1
Linamarase from Cassava (S35175)	1

Quinone oxidoreductase from *Triphysaria versicolor* (AF304461)	1
Oxalate oxidase (germin protein) from *A. thaliana* (AB009048)	1
Methionine sulfoxide reductase from *Fragaria ananassa* (Z69596)	1
Ferritin from Cowpea (AF052058)	1
Argonaute protein from *A. thaliana* (U91995)	1
Hev b3 from Rubber tree (AJ223388)	1

ESTs of known function but not involved in defence or stress responses

Similarity	Number of ESTs in library
Vegetative storage protein from Poplar (AF330050)	15
Major latex-like protein from *Prunus persica* (AF239177)	5
Amidase-like protein from *A. thaliana* (AL161586)	1
Desaturase from Castor bean (AF005096)	1
Digalactosyldiacylglycerol synthase from *A. thaliana* (AF149841)	1
DIP2 transcription enhancer from *A. thaliana* (AJ278493)	1
Late embryogenesis protein from *Prunus armeniaca* (AF071894)	1
Monodehydroascorbate reductase from *A. thaliana* (AC010927)	1
Omega-6 fatty acid desaturase from *Sesamum indicum* (AF192486)	1
Pectin methyl esterase	1
Ribosomal protein from *A. thaliana* (AC009465)	1
RNA helicase from *A. thaliana* (AC016661)	1
Short chain alcohol dehydrogenase from tobacco (AJ223177)	1
Xylan endohydrolase from *A. thaliana* (AB008015)	1
Protein similar to PHZF *A. thaliana* (AC004044)	1
Ankyrin from *A. thaliana* (AC016661)	1

ESTs of no known function

Similarity	Number of ESTs in library
Hypothetical protein from *A. thaliana* (AB023028)	2
Hypothetical protein from *A. thaliana* (AB010071)	1
Hypothetical protein from *A. thaliana* (AB015476)	1
Hypothetical protein from *A. thaliana* (AB018121)	1
Hypothetical protein from *A. thaliana* (AB023039)	1
Hypothetical protein from *A. thaliana* (AC003000)	1
Hypothetical protein from *A. thaliana* (AC003027)	1
Hypothetical protein from *A. thaliana* (AC003672)	1
Hypothetical protein from *A. thaliana* (AC006532)	1
Hypothetical protein from *A. thaliana* (AC009324)	1
Hypothetical protein from *A. thaliana* (AC022287)	1
Hypothetical protein from *A. thaliana* (AF370357)	1
Hypothetical protein from *A. thaliana* (AL035396)	1
Hypothetical protein from *A. thaliana* (AL161531)	1
Hypothetical protein from *A. thaliana* (AL163572)	1
Hypothetical protein from *A. thaliana* (AL163818)	1
Unknown gene from Sugar beet (AP000397)	1
No significant similarity found	1
No significant similarity found	1
No significant similarity found	1
No significant similarity found	1
No significant similarity found	1
No significant similarity found	1

No significant similarity found	1
No significant similarity found	1
No significant similarity found	1
No significant similarity found	1